The Emerging Roles of BAL1/ARTD9 in IFNs Signaling and Cancer

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SUMMARY

The B-aggressive lymphoma protein and ADP-ribosyltransferase BAL1/ARTD9 is a nucleocytoplasmic shuttling protein and belongs to the intracellular diphtheria toxin-related ARTD family (former PARPs) of mono- and polymerizing-ADP-ribosyltransferases. Expression of BAL1/ARDT9 is highly induced in most cells upon IFNγ, IFNα/β or LPS treatment. BAL1/ARDT9 has been initially identified as a potential risk-related gene product in aggressive diffuse large B-cell lymphoma (DLBCL). BAL1/ARDT9 is constitutively expressed in a subset of aggressive chemoresistant high-risk subtypes of DLBCL associated with an active but ineffective IFNγ mediated host inflammatory response (HR). A recent study provided preliminary evidence that BAL1/ARDT9 might be associated with lymphocyte migration. BAL1/ARDT9 has been proposed to promote the dissemination of malignant B cells in high-risk DLBCL and thus a potential novel target for chemotherapy. Our preliminary studies investigating the role of B-aggressive lymphoma protein and ADP-ribosyltransferase BAL1/ARTD9 in DLBCL and metastatic prostate cancer provide preliminary evidence that BAL1/ARDT9 is directly involved in modulation of IFNγ- dependent signaling and gene expression in HR-DLBCL and prostate cancers.

We found that BAL1/ARTD9 is constitutively expressed in DLBCL associated with constitutively active STAT1 signaling. Our results indicate that STAT1 could act as an oncogene in high-risk DLBCL with an active host inflammatory response. This activity is at least partially mediated by the B-aggressive lymphoma protein and ADP-ribosyltransferase BAL1/ARTD9. We could identify BAL1/ARDT9 as a novel IFNγ-dependent repressor of the tumor suppressor IRF1 and transcriptional activator of

BCL6 and IRF2. Strikingly, BAL1/ARDT9 inhibits IRF1 at the level of expression and nuclear translocation. Moreover we could show that BAL1/ARTD9 interacts with both STAT1α and STAT1β through its macro domains in a mono-ADP-ribosylation dependent manner. As a consequence, BAL1/ARTD9 represses IRF1/p53 dependent pro-apoptotic pathways and mediates proliferation and survival in high-risk diffuse large B-cell lymphomas. A similar observation was made in p53 negative metastatic prostate cancers cell lines suggesting that BAL1/ARTD9 negatively regulates IFNγ-dependent tumor suppressor genes and contributes to cell survival and tumor growth in different types of cancers.

ZUSAMMENFASSUNG

Das B-aggressive Lymphoma (BAL) Protein-1, auch ADP-ribosyltransferase ARTD9 genannt gehört zu einer Enzymfamilie von intrazellulären Diphtheria-Toxin-ähnlichen mono- und poly-ADP-ribosyltransferasen (ARTDs). BAL1/ARTD9 ist sowohl im Zellkern als auch Zytoplasma der Zelle lokalisiert. Die Expression von BAL1/ARTD9 ist sehr stark kontrolliert und wird hauptsächlich nach Stimulation der Zellen mit Interferonen (IFNγ, IFNα/β) und LPS aufreguliert. BAL1/ARDT9 wurde kürzlich als potentieller Risikofaktor in hochmalignen diffus großzelligen B-Zell-Lymphomen (DLBCL) identifiziert. Diese Studien zeigten, dass BAL1/ARDT9 in chemoresistenten Hochrisiko-DLBCL Formen konstitutiv überexprimiert ist welche eine aktive aber ineffektive IFNγ abhängige Immunantwort auslösen (HR-DLBCL). Diese Studien schlugen vor, dass BAL1/ARDT9 für die Zellmigration von B-Lymphozyten und Lymphomazellen verantwortlich sein könnte.

Unsere eigenen Studien deuten darauf hin, dass BAL1/ARDT9 direkt in der Regulierung von IFNγ abhängigen Signalkaskaden und Genexpressionsprogrammen involviert ist, sowohl in DLBCL als auch in Prostatakrebs. Konstitutive Überexpression von BAL1/ARDT9 in HR-DLBCL und Prostatakrebs korreliert direkt mit konstitutiven und IFNγ-abhängigen STAT1 Aktivitäten. Unsere Studien zeigen weiter, dass STAT1 in diesen Krebsformen nicht als Tumorsuppressor sondern vielmehr als BAL1/ARDT9 abhängiges Onkogenprodukt wirkt. Wir konnten BAL1/ARDT9 als IFNγ abhängigen Onkogen-ähnlichen Faktor in DLBCL identifizieren der einerseits den Tumorsupressor IRF1 inhibiert und gleichzeitig die Expression der beiden Onkogenprodukte BCL6 und IRF2 in aggressiven DLBCL Formen stimuliert. Bemerkenswerterweise inhibiert BAL1/ARDT9 nicht nur die transkriptionelle Aufregulierung von IRF1 sondern auch die Translokation von IRF1

in den Zellkern. Unsere Studien zeigen darüber hinaus, dass BAL1/ARDT9 mit beiden STAT1 Isoformen STAT1α und STAT1β interagieren kann. Die Interaktion findet über die Makro-Domänen von BAL1/ARDT9 statt und wird durch ADP-ribosylierung positiv reguliert. Die Inhibierung von IRF1 und Aufregulierung von BCL6 respektive IRF2 durch BAL1/ARDT9 führt nun dazu dass die pro-apoptotischen und anti-proliferativen Kaskaden unterdrückt werden. Als Konsequenz fördert BAL1/ARDT9 die Proliferation und das Überleben von chemoresistenten Hochrisiko-Formen von DLBCL und Prostatakrebs.

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ABBREVIATIONS

ABC DLBCL	Activate B cell-DLBCL
ADP	adenosine diphosphate
AMD	automodification domain
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
anti-CD20	Rituximab
ARH	ADP-ribose-protein hydrolase
ARTD	ADP-ribosyltransferase diphtheria toxin-like
ATM	Ataxia telangiectasia mutated
ATP	adenosine triphosphate
BAL	B-aggressive lymphoma protein
BBAP	Binding to B-aggressive lymphoma protein 1
BCR	B cell receptor
BCR-DLBCL	B-cell receptor/proliferation-DLBCL
BCL2	B-cell CLL/lymphoma 2
BCL6	B-cell CLL/lymphoma 6
BLIMP	B lymphocyte-induced maturation protein
BRCA1, 2	Breast Cancer gene 1,2
CaMKII	Ca(2+)/calmodulin-dependent kinase II
ChIP	chromatin immunoprecipitation
CHOP	cyclophosphamide, hydroxydaunorubicin, vincristine, and prednisone
CK2	Casein kinase-2
CPT	camptothecin
CTD	carboxy-terminal domain
DAPI	4',6-diamidino-2-phenylindole
DLB-CLS	Diffuse large B cell lymphoma
DLBCL-NOS	not otherwise specified-DLBCL
DTXL3	Deltex (DTX) E3 ubiquitin ligase 3 like
GCB- DLBCL	Germinal-Center DLBCL
HR	homologous recombination

HR-DLBCL	tumor microenvironment/host inflammatory response DLBCL
HSCs	Hematopoietic stem cells
IFN	Interferon
IFNγ	Interferon gamma
IL	Interleukin
IκB	NF-κB inhibitor IKK IκB kinase
IR	ionizing radiation
IRFs	Interferon responsive factors
JAK	Janus kinase
JNK1	c-Jun N-terminal kinase-1
LPS	lipopolysaccharide
(M)ARH	(mono)-ADP-ribose-arginine hydrolase
MSC	Mesenchymal Stem Cell
NAD+	nicotinamide adenine dinucleotide
NBS1	Nijmegen breakage syndrome 1
NC-DLBCL	not classified-DLBCL
NF-κB	nuclear factor κB
OCI-Ly	Ontario Cancer Institute Lymphoma
OXP-DLBCL	oxidative phosphorylation –DLBCL
p38-MAPK	p38 mitogen-activated protein kinase
p53/TP53	Tumor protein 53
PAR	poly(ADP-ribose)
PARG	poly(ADP-ribose) glycohydrolase
PARP	poly(ADP-ribose) polymerase
PG-DLBCL	primary gastric diffuse large B-cell lymphomas
PI3K	phosphatidylinositol-3 kinase
PKC-δ	protein kinase C delta
PM-DLBCL	primary mediastinal large B cell lymphomas
PTEN	Phosphatase and tensin homolog
PTM	post-translational modification

RAG	Recombination activating gene
STAT	signal transducer and activator of transcription
SUDHL	Southwestern University diffuse histiocytic lymphomas
T/HRBCL	T cell/histiocyte rich large B-cell lymphoma

INTRODUCTION

CHAPTER1.

1.1 Diffuse large B-cell lymphoma (DLBCL)

Diffuse large B-cell lymphoma (DLBCL) is a clinically and genetic heterogeneous non-Hodgkin's subtype of B-cell lymphoma and the most common lymphoid malignancy in adults, accounting for roughly 35% of all lymphoma cases worldwide (1-3). In the Western world, nearly 90% of aggressive mature B-cell lymphomas are identified as DLBCL (4, 5). This heterogeneous disease takes an aggressive and fatal clinical course with a median survival of less than one year if left untreated. Since the 1970s, overall incidence of non-Hodgkin lymphomas has increased about 45% in Switzerland, with an annual incidence of approximately 1500 cases per year, corresponding to an incidence of 4% of the total cases of new cancers in Switzerland per year (53.1% men) and (46.9% women) and to a mortality rate of 3.2% per year of total cancer death rate (http://www.krebsforschung.ch) (4, 5). Although more than half of DLBLC patients succumb to the disease, the rest of them can be cured with current multi-agent chemo-, radio- and/or immunotherapeutic regimens, representing one of the successes of modern cancer therapy (1-3, 6). However, over the last four decades, mortality from DLBCL increased in most developed areas of the world. DLBCL were among the few neoplasms showing consistent upward trends in North America and Europe over the last few decades (4, 5, 7).

Several genetic abnormalities including aberrant somatic hypermutation and translocations (i.e. BCL2, c-MYC or BCL6) and overexpression of BCL2/Rel/MYC, constitutive activation of STAT6, BCL2 and/or NF-κB pathways have been identified in DLBCLs (1-3). DLBCL is also often associated with defective apoptosis or DNA repair. DLBCL is thought to arise from normal antigen-exposed B cells, however the

exact cause and molecular mechanisms underlying these observations still remains unknown (1-3). It has been proposed that the initiating oncogenic event occurs early in B cell development but allows further differentiation to take place before subsequent oncogenic hits are sustained (**Fig.1**). The germinal center B cell is the normal counterpart of follicular lymphoma, Burkitt lymphoma, and most subtypes of diffuse large B cell lymphoma (DLBCL) (8-12) while the activated B cell-like (ABC) subtype of DLBCL resembles post–germinal center plasmablasts (6, 8, 9)(see also below). Other follicular lymphoma and subtype of DLBCL may also originate from a bone marrow pre-B cell even though the malignant cells phenotypically resemble germinal center B cells. It has been also suggested that many of the genetic lesions that initiate lymphomagenesis are aberrant by-products of the enzymes that rearrange Ig segments to assemble the B cell receptor (BCR) in normal B cells (6). These distinct translocations that defines the subtypes of DLBCL and other lymphoma such as follicular lymphoma are caused by the RAG recombinase, which is active in pre-B cells but not mature B cells indicating that these B cell lymphoma cells participated in a germinal center reaction (6, 13).

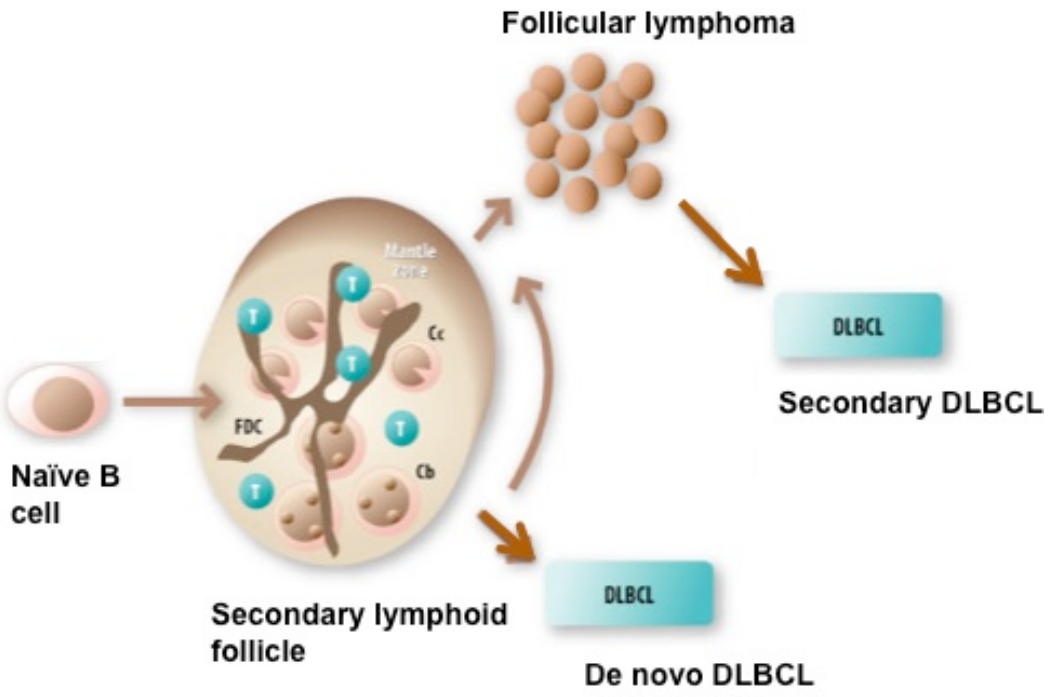

Fig.1 DLBCL cell development.

(From Staudt, L. M. & Dave, S., The biology of human lymphoid malignancies revealed by gene expression profiling Adv Immunol. 2005, (14)).

1.2 DLBCL subgroups and tumor classification

DLBCL have been originally divided in at least 5 molecularly and clinically distinct subgroups: DLBCL not otherwise specified (DLBCL-NOS), which is the largest group of DLBCL, primary mediastinal B cell lymphoma (PM-DLBCL), T cell/histiocyte rich large B-cell lymphoma (T/HRBCL), primary gastric diffuse large B-cell lymphomas (PG-DLBCL) and unclassifiable DLBCL with features intermediate between DLBCL and Burkitt lymphoma (DLBCL/BL) (8, 15-20). Burkitt Lymphoma (BL) is a highly aggressive B-cell non-Hodgkin's non-DLBCL lymphoma that mainly affects children and young adults. BL is associated with Epstein–Barr virus (EBV) in 80-95% of cases (11, 21). Interestingly, T/HRBCL, an

uncommon rare morphologic variant of DLBCL is characterized by distinct clinico-pathologic features, an active host immune/inflammatory response and increased expression of genes involved in T-cell receptor signaling, natural killer cell activation, complement cascade members and macrophage/dendritic cell markers.

Based on gene expression profile analysis and origins, the mayor group, DLBCL-NOS was recently reclassified into four biologically and clinically distinct subtypes: “germinal centre B-cell-like” (GCB)-DLBCL, “activated B-cell-like” (ABC)-DLBCL, primary mediastinal large B-cell lymphomas (PM-DLBCL) and a fourth group of DLBCL, which is represented by cases that remain non-classified, referred to as (NC)-DLBCL (2, 14-16, 19, 20). PM-DLBCLs are arising in the mediastinum of young adults, GCB-DLBCLs seem to derive from normal germinal center B cells (GC centroblasts), whereas ABC-DLBCLs may arise from post-germinal center B cells that are arrested during plasmacytic differentiation, though their cell of origin is not yet fully elucidated (2, 14-16, 19, 20). GCB-DLBCLs have ongoing somatic hypermutation of their immunoglobulin genes, a characteristic feature of normal germinal center B cells (22). The GCB-DLBCLs express genes characteristic of normal germinal-center B cells and are often associated with a good outcome. The GCB-DLBCL subtype is also characterized by low level of NF-κB activation and its survival is not dependent on NF-κB (23). On the other hand, key feature of the most aggressive DLBCL subtype, ABC-DLBCL, is the constitutive activation of nuclear factor kappa B (NF-κB)-dependent gene expression and its dependency on NF-κB activity for proliferation and survival (23). Moreover, ABC-DLBCL express genes characteristic of activated blood B cells and most of them are associated with a poor outcome (15, 20).

More recent genome-wide gene expression studies revealed the existence of three

additional discrete DLBCL subsets. Based on their unique transcriptional profiles and associated clinical and genetic features, GCB-, ABC- and NC-DLBCL were further subdivided in oxidative phosphorylation (OXP)-DLBCL, B-cell receptor/proliferation (BCR)-DLBCL, and tumor microenvironment/host inflammatory response (HR)-DLBCL (16, 19). The molecular profiles and clinico-pathologic features of HR-DLBCL tumors resemble those of T/HRBCL (16). Primary HR-DLBCL tumors are associated with increased expression of subsets of inflammatory mediators including up-regulation of interferon (IFN)-γ and NF-κB pathways (16, 24). The majority of HR-DLBCL belongs to the non-classified NC-DLBCL group, while smaller subsets belong to the ABC- and GCB-DLBCL (16, 19, 24, 25). Interestingly, the clinical outcome of the HR-DLBCL cluster is not improved, despite the increased inflammatory response (16, 19). Thus, it has been suggested that either their immune responses are inhibited by counter-regulatory mechanisms (i.e. through immuno-editing) or HR-DLBCL tumors were resistant towards chemotherapy, or a combination of both (16, 24). HR-DLBCLs are often associated with chemo-resistance (16, 19). Remarkably, HR-DLBCLs lack most of the common cytogenetic abnormalities (16, 19, 24, 25) and thus the exact molecular mechanisms underlying the oncogenic transformation and chemo-resistance in these tumors remain to be elucidated.

1.3. Oncogenic pathways in DLBCL subgroups

Transformation of normal B cell signaling pathways into malignant signaling pathways to sustain the growth and survival of DLBCL is accomplished through either gain-of-function mutations that activate signaling effectors or loss-of-function mutations that inactivate negative regulators of signaling or autocrine receptor

activation (26). One of the most compelling arguments supporting the view that the DLBCL subgroups represent distinct diseases is that they utilize distinct oncogenic mechanisms (Table 1). Insight into the molecular mechanisms underlying of the clinically distinct features of DLBCL subgroups has been provided by an analysis of regulatory factors that control the differentiation of germinal center B cells to plasma cells. As mentioned above the observed genetic abnormalities (i.e. translocation) often result in over-expression and constitutive activation of STAT3, STAT6, BCL2, BCL6, Rel factors and/or c-Myc, in DLBCLs (1-3).

For instance, BCL6 is a transcriptional repressor that is required for mature B cells to differentiate into germinal center B cells during an immune response (27). Normal germinal center B cells express BCL6 at high levels but BCL6 expression is silenced during plasmacytic differentiation (28);(29);(30). In contrast the proto-oncogene gene product BCL6 is over-expressed in the majority of patients with aggressive DLBCL (31). Most DLBCLs belonging to the GCB subgroup constitutively express BCL6 at very high levels (8, 9, 20). Constitutive expression of BCL6 mediates lymphomagenesis through aberrant proliferation and cell survival (31). BCL6 can suppress both the basal and the inducible transcription of the tumor suppressors p53, p21 and or BLIMP1 in DLBCL (31-34). BCL6 gene is deregulated by chromosomal translocations in roughly 20% of DLBCLs (35) but the high constitutive expression of BCL6 in GCB-DLBCLs is not accounted for by these translocations. Rather, BCL6 is constitutively expressed in GCB-DLBCLs along with other germinal center B cell restricted-genes because these DLBCLs are derived from normal germinal center B cells and retain much of their biology (26).

Another oncogenic translocation event occurs at the BCL2 locus. The t(14;18) translocation deregulates the BCL2 gene by placing it near the enhancer elements of

the immunoglobulin heavy chain locus. This oncogenic event was found to be common in GCB-DLBCL, occurring in about 45% of cases analyzed, and was also detected in about 20% of PM-DLBCL cases (2, 20, 36). In contrast, no BCL2 translocations had been found so far in ABC-DLBCLs (2, 20). Nonetheless, the majority of ABC-DLBCLs express BCL2 mRNA at high levels, presumably due to transcriptional deregulation of the BCL2 gene (2, 9, 20).

	GCB DLBCL	ABC DLBCL	PMBL
c-rel amplification	16%	0	25%
BCL-2 translocation	45%	0	18%
Gain Chr. 3q	0	24%	5%
Gain/amp Chr. 9p24	0	6%	43%
Constitutive NF-κB Activation	–	+	+

Table1. Distinct oncogenic mechanisms in the DLBCL subgroups
(from Staudt, L. M. & Dave, S.,, The biology of human lymphoid malignancies revealed by gene expression profiling Adv Immunol. 2005, (14)).

Many oncogenic events in B-cell lymphomas converge on the anti-apoptotic NF-κB pathways, often associated with over-activated survival signaling provided by the phosphatidylinositol-3 kinase (PI3K) and JAK kinase pathways (26, 37). Indeed, ABC-DLBCLs were found to have high expression of known NF-κB target genes when compared with GCB-DLBCLs (37). Remarkably the observed high constitutive activity NF-κB pathways in most ABC-DLBCLs are not connected to any amplification or translocations of the *Rel*-loci (26, 37). One of the most important differences among the DLBCL subgroups is the constitutive activity of the NF-κB pathway in ABC-DLBCL and PM-DLBCL but not GCB-DLBCL (26, 37). In this respect, PM-DLBCLs resemble Hodgkin lymphoma, which is also characterized by constitutive NF-κB activity (38-42). On the other hand, the amplification of the *c-rel*

locus on chromosome arm 2p occurs in 16% of GCB-DLBCLs and in 25% of PM-DLBCLs, but has never been detected in ABC-DLBCLs (2, 20, 43). C-Rel is a member of the anti-apoptotic NF-κB family of transcription factors. Nonetheless, this translocation does not result in constitutive expression of NF-κB factors in the majority of GCB-DLBCLs (26, 37).

Together, the uneven distribution of these chromosomal abnormalities among the DLBCL subgroups suggests that the subgroups utilize distinct oncogenic pathways. More over, DLBCL simultaneously often activate several interconnected signaling pathways, resulting in distinct clinical outcomes, but at the same time also providing therapeutic opportunities and challenges.

1.4. Clinical outcome and DLBCL lymphoma/ cancer therapies

While tremendous progress has been made in understanding the molecular mechanisms underlying the biological heterogeneity in highly malignant aggressive DLBCL, much remains unknown about the molecular (patho-physiological) mechanisms, which determine the clinical outcome. It is therefore important to get more insight into the molecular mechanisms underlying their patho-physiology and to identify new targets for novel therapeutic treatments. Currently, chemotherapy (CHOP; cyclophosphamide, hydroxydaunorubicin, vincristine, and prednisone), combined with radiation and/or the anti-CD20 antibody Rituximab is the mainstays of lymphoma treatment. Optimal cancer therapies target the abnormal state of the cancer cell while sparing normal cells. Ideally, one hopes to identify and exploit “synthetic lethality” with therapies that target the oncogenic rewiring of malignant cells (44, 45). Although more than half of DLBCL patients can survive with current (immuno)-chemotherapeutic regimes, the rest of them succumb to the disease. Thus, for the

remainder of these patients novel therapeutic strategies are required. Why some patients with DLBCL can be cured and others not has been a longstanding and is still being a frustrating open question. The recent gene expression profiling studies in clinically distinct classes of high risk DLBCLs revealed that signaling pathways might serve as novel (immuno)-chemotherapeutical targets in DLBCL, (2, 15, 19, 20). The constitutive activation of signaling pathways in lymphoma offers opportunities to intervene with tolerable effects on normal immune function. Moreover, B cell–restricted transcription factors that are essential for lymphoma survival may also be amenable to attack because a short-term loss of normal B cells can be managed without risk to the patient. Indeed recent findings suggest that NF-κB pathways are potential therapeutic targets specific for ABC-DLBCL and PM-DLBCL, but not GCB-DLBCL. In support of this idea, small molecule inhibitors of inhibitor of κB (IκB) kinase were found to be selectively toxic for ABC-DLBCL and PM-DLBCL cell lines, but had no effect on GCB-DLBCL cell lines (46). In addition, the activated signal transducer and activator of transcription (STAT)-3 dependent signaling pathway can also serve as novel drug target in DLBCL (46-49). In contrast to normal cells, in which STAT phosphorylation occurs transiently, it has been determined that STAT3 is persistently phosphorylated in most ABC-DLBCL in a autocrine and paracrine manner (from the tumor microenvironment) (47). Indeed, small molecule inhibitors of STAT3 signaling (nuclear translocation) were found to be selectively toxic for ABC-DLBCL (46, 48, 49). Recent studies provided also evidence that small molecule inhibitors targeting BCL6 can be selectively toxic towards high risk GCB-DLBCL constitutively expressing BCL6 (50-53). Moreover, several genes, including the B-aggressive lymphoma protein 1 (BAL1) and BAL1-binding protein (BBAP) and Deltex E3 ubiquiton ligase 3 like (DTX3L) have been recently identified in a genome-

wide screen for risk-related genes in high risk DLBCL (54, 55). For instance, BAL1 and DTX3L are highly expressed in chemo-resistant HR-DLBCL with an active IFNγ mediated host inflammatory response but not in cured low-risk tumors (25, 54, 55).

1.5. Prostate cancer

Prostate cancer is one of the leading causes of cancer-related mortality and morbidity in the aging male population and representing the most frequently diagnosed malignancy in men around the world (56, 57). Prostate cancer is a common clinically and molecularly heterogeneous disease and it is characterized by its aggressive metastasis. Similar to leukemias and lymphomas at least 50% of prostate cancers harbor recurrent gene rearrangements reflecting the distinct biology to prostate cancer subtypes including the aggressiveness of disease (56, 58-60). A common feature of many prostate cancer subtypes is the dependence on NF-κB and the activated signal transducer and activators of transcription (STAT)-3 and STAT6 for survival (57). Prostate cancer displays different stages and grades related to the aggressive metastasis disease (56, 57). Current therapeutic approaches for prostate cancer include active surveillance, surgery, radiation therapy, hormone therapy, chemotherapy and immunotherapy (57). The observed clinical and molecular heterogeneity associated with this common disease is still a mayor challenge in understanding prostate cancer (57-60). Most cases of clinically localized prostate cancer are curable with effective surgical and radiation treatments i.e. when the cancer is contained within the prostate (56, 57). Though, a significant percentage of patients with localized prostate cancer have radiation-resistant disease. Approximately 80% of all prostate cancer cases diagnosed are in early stages at which the therapeutic options are mostly curative.

However, the mortality rate of the remaining 20% of cases, diagnosed as metastatic tumors, is very high (56, 57).

Patients diagnosed with prostate cancers and de novo metastatic tumors are generally treated with androgen deprivation therapy since the growth of prostate cancer is originally androgen dependent (56, 57). However, androgen deprivation therapy is primarily palliative, nearly all patients will eventually develop the androgen-independent and highly metastatic form of prostate cancers (56, 57). Unfortunately, chemo-resistance remains the major obstacle in the treatment of androgen-independent prostate cancer (57-60). Thus, novel therapeutic approaches to impede or prevent the progression of the disease remains to be developed for androgen-independent prostate cancer, especially for treating chemo-resistant prostate cancers**.**

1.6 IFN/JAK-STAT1/2 signaling pathways

Interferons (IFN) encompass a family of secreted and pleiotropic cytokines that exhibit distinct antiviral, antiproliferative, immunomodulatory and antitumor properties (61-63). They are grouped into three classes called type I, II and III IFNs (61-63): Type I interferons consist of INFα, INFβ, IFNδ, IFNσ, IFNε, IFNκ and IFNτ. INFα, INFβ and IFNδ, and are produced by nearly all cells, which get infected by viruses (61-64). IFNα is mainly produced by certain leukocytes (such as plasmacytoid dendritic cells and macrophages), while IFNβ mainly produced by epithelial cells and fibroblasts (61-65). Knockout mice studies demonstrated that the type I interferons IFNα, IFNβ and IFNδ are primarily antiviral and have just little immunomodulatory activity but are essential for protective immunity to experimental infection by numerous viruses (61-65). The other type I interferons IFNσ, IFNε, IFNκ and IFNτ play less well-defined roles, such as regulators of maternal recognition in

pregnancy (61-65). Type I interferon system plays a key role in shaping the antiviral adaptive immune response. Type II Interferon consists of one single interferon, namely INFγ, which is produced only by the specialized and mitogenically activated T-cells and natural killer (NK)-cells. Its antiviral effects are less distinct than the one of type I, because its main effect is immuno-modulation. On the other hand type II interferon is critical for protective immunity to a number of intracellular bacteria, fungi, and parasites (61-65). Type III Interferon is also called INFλ and produced by many cells. Its antiviral and immunomodulatory effect is weaker than the ones of type I or II (61-65).

The IFN systems are well-controlled networks that require the coordinated regulation of immediate signaling events, rapid transcriptional activation and post-transcriptional control. The biological effects of IFNs are primarily mediated through the activation of the signal transduction pathway of the Janus kinases (JAK1-4) and signal transducers and activators of transcription (STAT) proteins, which in turn results in subsequent induction of IFN dependent genes (66-68). STAT proteins comprise a family of transcription factors (STAT1α, STAT1β, STAT2, STAT3α, STAT3β, STAT3γ, STAT4α, STAT4β, STAT5α, STAT5β, and STAT6α, STAT6β, STAT6γ, **Fig.2**) that are essential for mediating cytokine- and growth factor-dependent cellular differentiation, proliferation, cell survival and apoptosis and immune function (68-72). Genetic evidence indicates that the three STAT proteins STAT1 and STAT2 play a mayor role in immune function. STAT1-deficient mice exhibit a selective signaling defect in response to interferons (69-71)). Stat1(-/-) mice exhibit selective signaling defects in their response to both type-1 and type-II IFNs (70, 73, 74). Several reports demonstrated that Stat1(-/-) and Stat2(-/-) mice are highly sensitive to infection by microbial pathogens and viruses (69-71, 73-77).

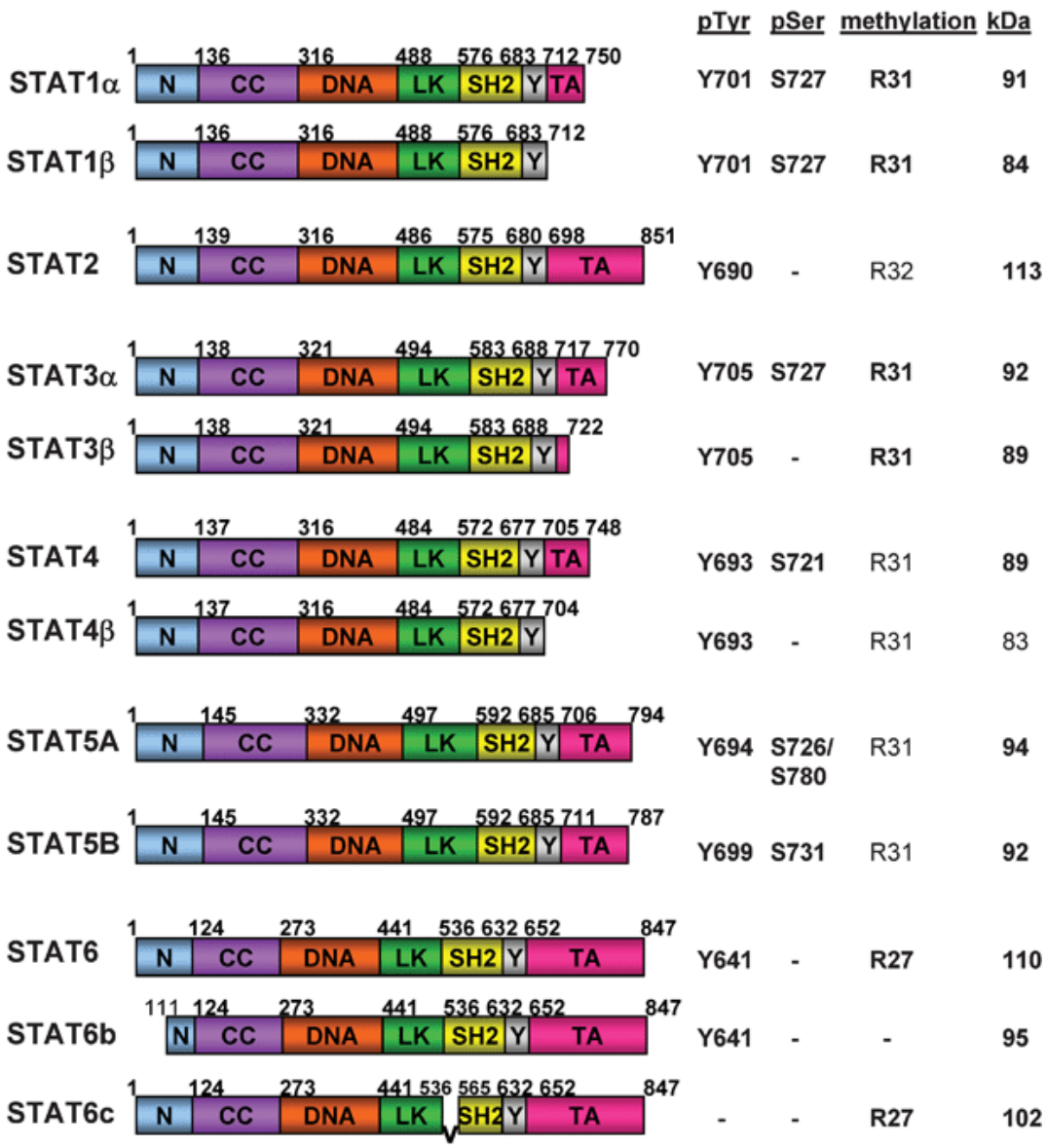

Fig.2 Domain architecture of the STAT family and their isoforms.
N: N-terminal dimerization/tetramerization domain, CC: coiled-coil domain involved in interaction with other proteins, DNA = DNA binding domain, SH2: SH2 domain, Y: phosphotyrosyl tail segment, TA: transactivation domain. The Y and S residues involved in phosphorylation of the proteins are listed. *(From Lim, C. P. & Cao, X. Structure, function, and regulation of STAT proteins, Mol Biosyst 2006 (72)).*

Of the known JAKs and STATs, the JAK1, JAK2, and TYK2 kinases and the STAT1 and STAT2 transcription factors play central roles in mediating IFN-dependent biological responses, including induction of the antiviral state (67, 70, 78, 79). IFN signaling is initiated through (IFNx) ligand interaction with specific trans-membrane receptor subunits of their corresponding receptors (**Fig.3**). Type I interferons (IFNα, IFNβ, IFNδ, IFNκ and IFNε) bind to a common type-I IFN receptor universally expressed on the cell surface, whereas IFNγ uses a distinct receptor-complex (Type-II IFN receptor) for signaling that is not present on all cell types (67, 68, 78, 79). The subsequent receptor oligomerization leads to activation of either an intrinsic kinase domain or receptor associated JAK kinases, JAK1 and TYK2 kinases function in IFNα/β signaling, and the JAK1 and JAK2 kinases function in IFNγ signaling. TYK2 interacts with the IFNAR-1 receptor subunit, and JAK1 interacts with the IFNAR2 subunit of the IFNα/β receptor. JAK1 also interacts with the IFNGR1 receptor subunit, and JAK2 interacts with the IFNGR2 subunit of the IFNγ receptor (67, 78, 79). The subsequent JAK-mediated tyrosine phosphorylation of latent cytoplasmic STAT proteins, resulting in STAT dimerisation via their Src homology (SH2) domain, translocation to the nucleus, complex formation with other transcription factors and binding to specific sequences to regulate gene transcription (66-68, 78, 79). Each type of IFN activates different combinations of STAT proteins (78). IFNγ triggers the phosphorylation of STAT1 but not STAT2. Activated STAT1 forms homodimers and migrates to the nucleus, where it binds to GAS sequences present in many promoters of primary IFN response genes (68, 78, 79). On the other hand upon IFNα/ß stimulation, both STAT1 and STAT2 are phosphorylated and activated and consequently heterodimerize and translocate to the nucleus, where they associate with IFN regulatory factor 9 (IRF9/p48) to form a STAT1:STAT2:IRF9 trimeric

transcriptional complex (ISGF3) (68, 78, 79). This trimer then binds to cis-acting IFN-stimulated response elements (ISREs) to induce transcription of primary IFN response genes (ISGs). The IFN response comprises a multi-array of IFN-stimulated gene products. Oligonucleotide microarray studies have shown that up to 700 genes are induced following treatment with IFNs depending on the cell type and conditions (63, 80). One of primary IFN response genes is the transcription factor and interferon regulatory factor (IRF)-1, which in turn enhances transcription of several secondary response genes. IRF1 acts aside with two other members of the interferon regulatory factor (IRF) family - IRF3 and IRF7 - as a major modulator of IFN-dependent gene expression (81).

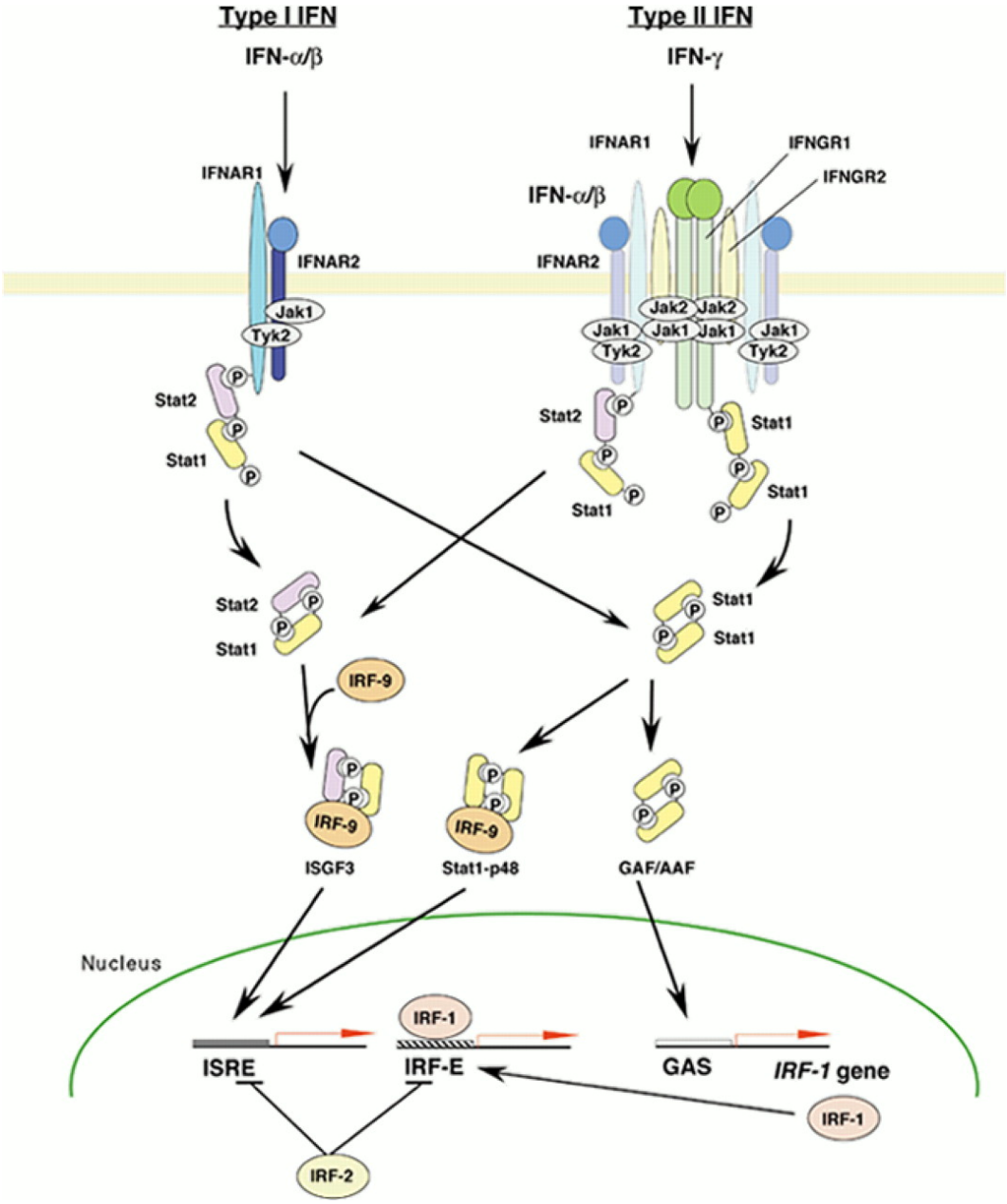

Fig.3 IFN signaling pathways
(Taniguchi T et al., IRF family of transcription factors as regulators of host defense, Annu Rev Immunol. 2001, (81)).

1.7. Functional roles of the IL-4/STAT6 pathway

The IFNγ/STAT1-signaling pathway can also be antagonistically regulated by other STATs, mainly by STAT6 (69-71). Studies on knockout mice have demonstrated that

STAT6 is specifically activated in response to IL-4 and IL-13, another cytokine that binds to the chain of the IL-4 receptor (82). STAT6 is tightly connected to IL-4 and IL-13 signaling, and plays a key role in Th2 polarization of the immune system (69-71). STAT6 plays an essential role in IL-4 signaling. Several reports showed that Stat6(-/-) mice have defects in IL-4- and IL-13-mediated functions including induction of CD23 and MHC class II expression, immunoglobulin class switching to IgE, B- and T-cell proliferation, decreased nitric oxide production of activated macrophages, Th2 cell development and thus have a reduced Th2-mediated immune response (69-71). On the other hand, they have elevated levels of IL-2 and showed an increased Th1-mediated immune response (69-71). Th2 cells produced IL-4 is an important negative regulator of proinflammatory gene expression. For instance, IL-4 is able to suppress transcriptional activation of IFNγ/STAT1-responsive pro-inflammatory genes through the ability of STAT6 to sequester coactivator molecules that may be required for the transcriptional action of STAT1 (82-86). On the other hand, IFNα/β and IFNγ have been shown to suppress IL-4/IL-13-inducible gene expression by inhibiting tyrosine phosphorylation and nuclear translocation of STAT6, for example in monocytes and fibroblast cells (82-86). Recent reports demonstrated that IFNα/ß can also activate complexes containing STAT6, predominantly in B cells and hepatocytes (87). Activation of STAT6 by IFNα/ß in B cells is accompanied by the formation of novel STAT2:STAT6 complexes (87). Thus, the intracellular mechanisms that mediate these antagonistic responses appear to be multifactorial.

1.8 Regulation of IFN/STAT1/2 signaling pathways

The interferon system is a well-controlled network that requires the coordinated regulation of immediate signaling events, rapid transcriptional activation, and post-transcriptional control. Stringent control of the IFN system allows swift elimination of tumor cells and pathogens and beneficial immunomodulatory functions prior to the onset of deleterious consequence for the host. Such controls offer a fine-tuning mechanism for efficient and rapid response and as a negative feedback control in IFN biosynthesis and response. The post-transcriptional mechanisms occur at multiple levels, including mRNA stability, alternative splicing, translation, and post-translational modifications (63). The threshold, magnitude and specific responses elicited by IFN stimulation are tightly controlled and mainly negatively regulated by numerous inducible negative feedback mechanisms including tyrosine phosphatases, receptor internalization, proteasomal degradation of signaling adaptor molecules, soluble receptor antagonists and specific inhibitors, including the suppressor of cytokine signaling (SOCS) proteins (reviewed in (88)). SOCS proteins have been revealed as key negative regulators of cytokine and growth factor signaling that can interfere with signaling not only from the inducing cytokine in a classic "negative-feedback" loop, but also to regulate signaling downstream of other cytokines. SOCS proteins inhibit components of the cytokine-signaling cascade via direct binding or by preventing access to the signaling complex. SOCS1 has been shown be the key SOCS protein playing an essential regulatory role in innate immunity (88). SOCS1 can interact with phosphotyrosine residues on the IFNAR1 and IFNGR1 subunits in a JAK1-independent manner, thus abrogating tyrosine phosphorylation of transcription factor STAT1 and reducing the duration of IFN dependent gene expression. In

addition, SOCS can block signaling by direct inhibition of JAK enzymatic activity (reviewed in (88)).

1.9 IFN/STAT1 signaling pathway in tumorigenesis

Genetic studies demonstrated that the type I interferons IFNα and IFNβ as well as the single type II interferon IFNγ play a mayor role as central coordinators of tumor-immune-system interactions (61, 63, 89). IFNγ plays a pivotal antitumor role since IFNγ or IFNγ receptor knockout mice show increased tumor development.

IFNγ promotes direct tumor suppressive effects by inhibiting cellular proliferation, promoting apoptosis and inhibiting angiogenesis (90, 91). Paradoxically, IFNs have been shown to exhibit both pro- and antitumor properties (63, 89, 92). IFNs are suggested to modulate the host response to tumors in two different ways. Initially, IFNs aid protecting the host from tumor formation and development (immuno-surveillance), but subsequently IFNs could also promote the tumors to resist attack (63, 89, 92). The pressure to recognize and eliminate all precancerous or cancerous cells by cells of the innate and adaptive immunity enhances the outgrowth of new mutated tumor variants with reduced immunogenicity that can escape the immune system and favor progression to detectable malignancies (immuno-editing) (92). Type I and Type II IFNs play a central role in the process of cancer immuno-editing (93, 94). The constant presence of type I and/or type II IFNs in the microenvironment, can result in a selection of tumor cells, which are no longer sensitive to interferons (20). Moreover, subsets of these tumor cells, can intrinsically produce constitutively interferons, and have also upregulated STAT1 and/or STAT2 (20). The tumor suppressor STAT1 is considered a key regulator of the surveillance of developing tumors. Deregulation of STAT signaling pathways is often associated with solid

tumors and leukemia (68, 78). STAT1 and STAT4 are mainly acting as tumor suppressor genes and their inactivation contributes to malignant transformation while the other STATs STAT3, STAT5 and STAT6 have been suggested to have mainly oncogenic functions and promoting survival of tumor cells (reviewed in (95-97)). For instance, Stat6(-/-) mice have enhanced immunosurveillance against primary and metastatic tumors since Stat6(-/-) mice are unable to generate a (IL-4 mediated) type 2 immune response, and instead mount an enhanced (IFN mediated) type 1 response (98, 99). Moreover, constitutively activated STAT6 has been found in patient samples isolated from prostrate cancer tissues, Hodgkin lymphomas, primary mediastinal large B cell lymphomas and T cell leukemia/lymphomas (100, 101). Both STAT6 and STAT1 have been suggested to act as crucial antagonistic regulators in the pathogenesis of solid tumors and leukemias (68, 78).

Remarkably, recent studies revealed that STAT1 acts both as a tumor suppressor and oncogene depending on the context and cancer type (102, 103). For instance loss of STAT1 in mice predisposes to intraepithelial neoplasias (104). On the other hand Stat1(-/-) mice are partially protected from leukemia development, indicating that STAT1 acts as a tumor promoter for leukemia development (105). The role of STAT2 in tumorigenesis is not yet clear but recent Stat2(-/-) mice model studies identified STAT2 as a novel contributor to colorectal and skin carcinogenesis that may act to increase the gene expression and secretion of proinflammatory mediators, which in turn activate the oncogenic STAT3 signaling pathway (106).

The tumor suppressive effects of IFNγ are mainly mediated through one of the mayor target of STAT1, the interferon regulatory factor-1 (IRF1). IRF1 and its functional antagonist IRF2 are not only mayor regulatory factor in innate immunity involved in the regulation of normal haematopoiesis and leukaemogenesis (107, 108). Beside its

crucial role in innate immunity IRF1 can also act as tumor suppressor and mediates anti-proliferative and pro-apoptotic effects in cancer cells in a context dependent and cell type- specific manner (81, 109-112). IRF1 is critical for IFNγ-mediated immune surveillance (113, 114). Many tumors lacking IRF1 or have reduced IRF1 expression levels (111, 112). Although loss of IRF1 alone seems not to be associated with spontaneous tumor development in mice, it greatly increases tumor susceptibility in combination with loss of other tumor suppressor proteins such as p53 (111). In absence of antagonistic regulatory factors, such as the oncogene IRF2 increased expression and activation of IRF1 inhibits the expression of pro-survival members of the BCL2 family and simultaneously induces the expression and activation of anti-proliferative and pro-apoptotic genes, including cyclin-dependent kinase (CDK) inhibitor p21WAF1 or CASP3 (107, 115, 116). IRF1 induces both ligand-dependent (extrinsic) and ligand-independent (intrinsic) caspase-mediated apoptosis (110). Finally a recent study suggests that IRF1 is also an essential mediator of the crosstalk between tumor cells and NK cells that mediate immune surveillance in the metastatic niche (113, 114).

1.10 Chemo- and radioresistance in cancer and the IFN/STAT1 signaling pathway

Several recent studies strongly indicate that transcription factor STAT1 is involved in chemo and/or radiation resistance of solid tumors (117-122). For instance, 29% of clinical human prostate cancers analyzed, constitutively expressed STAT1 and interferon stimulated genes (ISGs) in vivo (118). STAT1 has been therefore suggested as a potential target for radiosensitation of aggressive tumors that constitutively over-express IFNγ/STAT1-dependent pathways. It has been shown that multiple small

doses of RI can activate an IFN(α, β and γ)-related, STAT1-dependent DNA damage gene expression signature (including STAT1, G1P2, G1P3, IFITM1, IFIT1, IRF9, MX1, HLA-C, OAS1 and OAS3) in prostate cancer (117-121). Moreover, in vitro selection against IFNα or IFNγ and constitutive expression of STAT1 leads to an IFN- and radioresistant phenotype in prostate tumor cells (117, 118). In addition, the IFN/STAT1 signaling pathway is also up-regulated in chemo-resistant prostate cancer cells (118, 119). Remarkably, STAT1-dependent chemo-resistance was also associated with increased resistance to ionizing radiation and accompanied by the up-regulation of ISGs that overlapped, in part, with the IFN/DNA damage gene expression signature (117, 118). However, the exact molecular mechanisms how STAT1 signaling pathways mediate chemo- and radio-resistance in prostate cancer (**Fig. 4** shows the current model) and potentially in other cancer types remains to be elucidated.

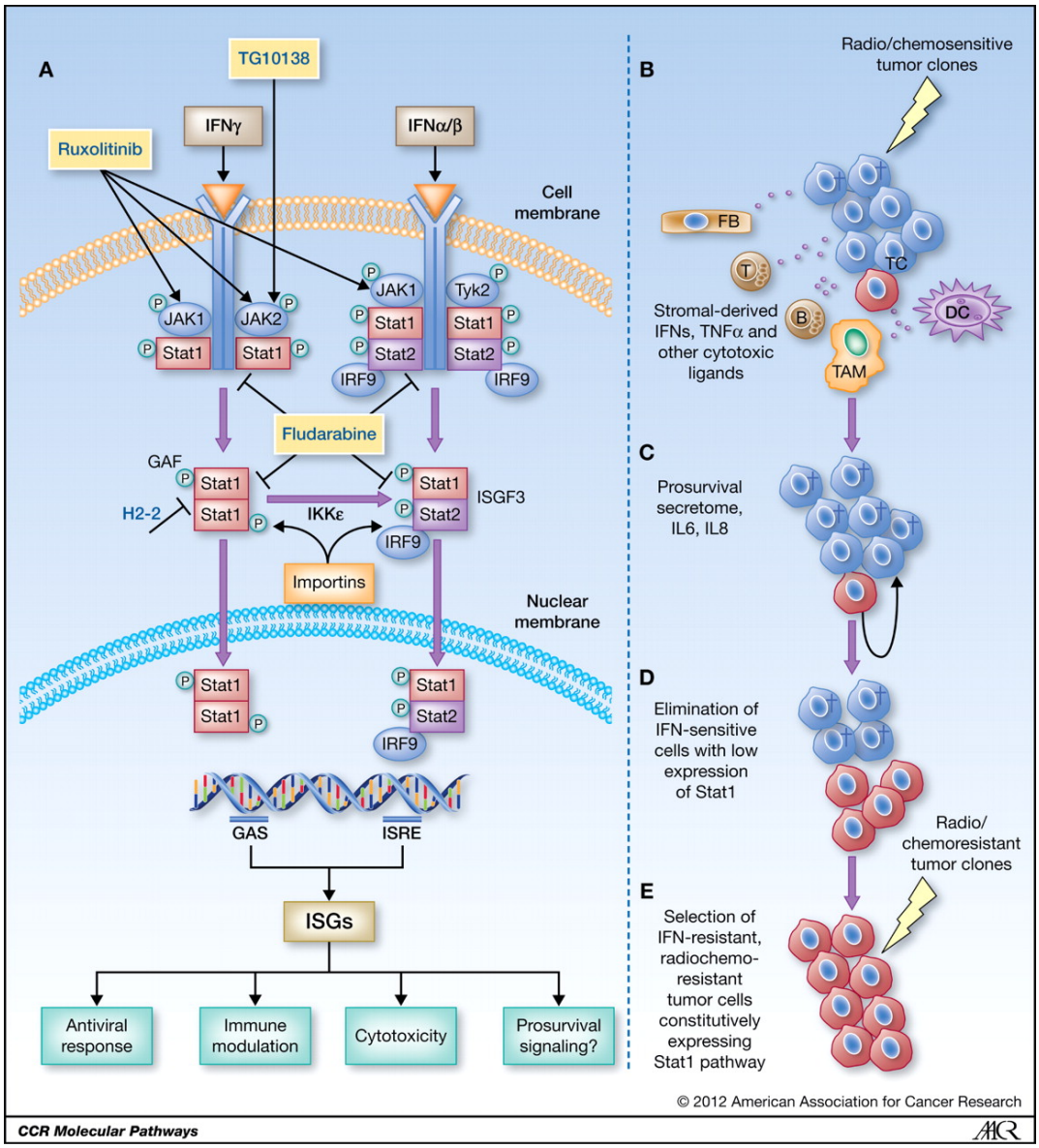

Fig.4 Radio and chemoresistance and the IFN/STAT1 pathway
(from N.N. Khodarev et al., Clin Molecular pathways: interferon/stat1 pathway: role in the tumor resistance to genotoxic stress and aggressive growth, Cancer Res, 2012, (118)).

1.11 STAT6 signaling pathways and chemo- and radioresistance in cancer

Aside the dual role of IFNs in tumorigenesis, recent studies demonstrated that aberrant activation of IL-4 receptor signaling and constitutively activated IL-4/STAT6 target genes is frequently associated with the pathophysiology of leukemia and

lymphoma cells (89). However, less is known about the tumorigenic roles of the STAT6, especially regarding chemo- and radioresistance. STAT6 is highly expressed in subtypes of prostate cancer, DLBCL and chronic lymphocytic leukemia (CLL) and its expression significantly correlated with tumor size in vivo (101, 123, 124). The IL-4/STAT6 signaling pathway has been suggested to be required for survival and induces proliferation and migration in prostate cancer under stress (101). Interestingly, recent studies showed that IL-4/STAT6 signaling pathway induced broad resistance to the cytotoxic drugs in chronic lymphocytic leukemia (CLL)(124), thus providing preliminary evidence that IL-4/STAT6 signaling is also involved in chemo-resistance of tumor cells.

CHAPTER2.

2.1 ADP-ribosylation reaction:

One of the most dynamic ways to regulate signaling and transcriptional processes is through post-translational modifications (PTMs) of proteins, such as phosphorylation, acetylation, methylation, ubiquitination or (n)ADP-ribosylation. PTMs are fundamental to the regulation of the physiology, the behavior, and the fate of cell. Among these modifications, ADP-ribosylation is both an established and a rapidly emerging PTM involved in a variety of essential cellular processes (125-127). Mono- and polymerizing-ADP-ribosylation of proteins are phylogenetically ancient, reversible, covalent post-translational modifications implicated in a wide range of processes (**Fig. 5**) (126). Mono-ADP-ribosylation of proteins was originally identified as the pathogenic mechanism of certain bacterial toxins, which function as virulence factors (126, 128, 129). Intracellular (n)ADP-ribosylation has been suggested to play

important roles in the regulation of intracellular signaling cascades, gene expression, as well as cell differentiation and proliferation (125, 126, 128, 130, 131). However, nothing is known about the regulation of IFN/STAT1 dependent signaling through mono or poly-ADP ribosylation. Interestingly, a recent report provided first evidence that IL-4-signaling and STAT6-mediated transcriptional responses might be indeed regulated through mono-ADP-ribosylation of transcriptional coactivators of STAT6 such as p100 (132).

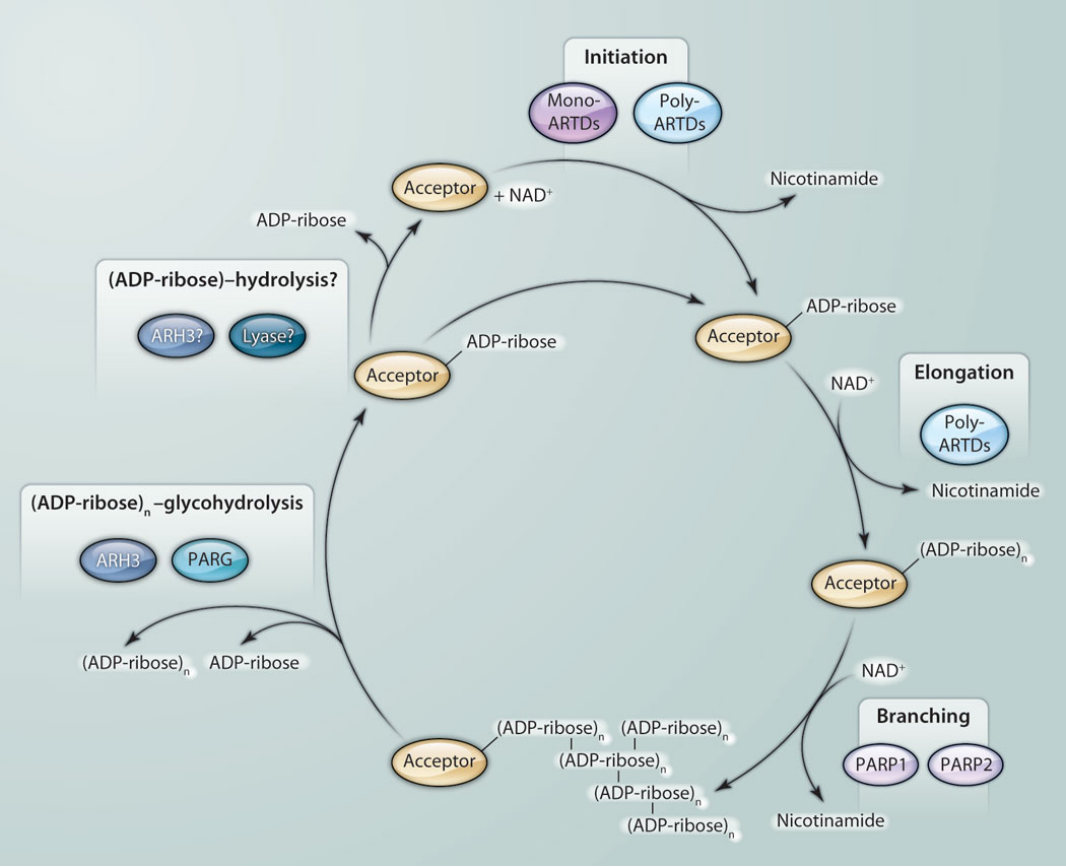

FIG.5 The ADP-ribosylation reaction cycle
(From Hottiger et al., Progress in the function and regulation of ADP-ribosylation, Sci. Signaling, 2011 , (133)).

2.2 The Diphtheria toxin-related ADP-ribosyltransferase (ARTD) family

The mono-ADP-ribosylation reactions are catalyzed by mono-ADP-ribosyltransferases (ARTs), which transfer a single ADP-ribose moiety of NAD+ to a specific amino acid residue (i.e. on glutamate residues) of the acceptor protein (126). The related polymerizing-ADP-ribosylation reaction catalyzed by polymerizing-ADP-ribosyltransferases (former poly-ADP-ribose-polymerases, PARPs) includes, in addition to the mono-ADP-ribose transfer reaction, an elongation step that results in the synthesis of poly-ADP-ribose chains processes (125) (126) (**Fig. 5**). In eukaryotic cells, cellular protein-mono-ADP-ribosylation reactions that modify arginine, glutamate and potentially cysteine residues of acceptor proteins have been detected in vivo (reviewed in (126, 134). In eukaryotes, mono-ADP-ribosylation of arginine and cysteine residues seems to occur on both extracellular and intracellular target proteins while (n)ADP-ribosylation of glutamate residues seems to be restricted to the nucleus and cytoplasm (126).

Currently, more than 30 human genes encoding proteins that possess either an ADP-ribosyltransferase or ADPr-protein(glyco)hydrolase catalytic domain are known. The only family of intracellular ADP-ribosyltransferases characterized so far encompasses the Diphteria toxin-like mono- and polymerizing-ADP-ribosyltransferases (ARTDs, former PARPs) (125-127, 135). It has been recently demonstrated that several identified intracellular mammalian Diphtheria toxin-related ADP-ribosyltransferases (such as the ARTD7/BAL3, ARTD8/BAL2, ARTD10, ARTD12 and ARTD15) exclusively functions as mono-ADP-ribosyltransferases (127, 136). Thus, the mammalian Diphtheria toxin-related ADP-ribosyltransferases were recently grouped under a new, unified nomenclature, referred to as ARTDs (135). The mammalian ARTD family encompasses 18(17) members (**Fig. 6**). Based on recent structural and

enzymological analysis the ARTDs can be divided into four mayor groups: (1) mono-ADP-ribosyltransferases, (2) polymerizing-ADP-ribosyltransferases, (3) inactive mono-ADP-ribosyltransferase enzymes, including BAL1/ARTD9, and (4) most likely also inactive polymerizing-ADP-ribosyltransferases such as ARTD3 or ARTD4 (137). The mono-ADP-ribosylating members of the ARTD family are very likely representing a missing link and are responsible for most mono-ADP-ribosylation reactions in mammalian cells. The ARTD family can be further subdivided into different subclasses based on their activity and overall domain structures. One of these subclasses includes the active and inactive macro domain-containing mono-ADP-ribosyltransferases and B-aggressive lymphoma proteins BAL1/ARTD9, BAL2/ARTD8 and BAL3/ARTD7, described as novel risk-related proteins in diffuse large B-cell lymphomas (127, 137).

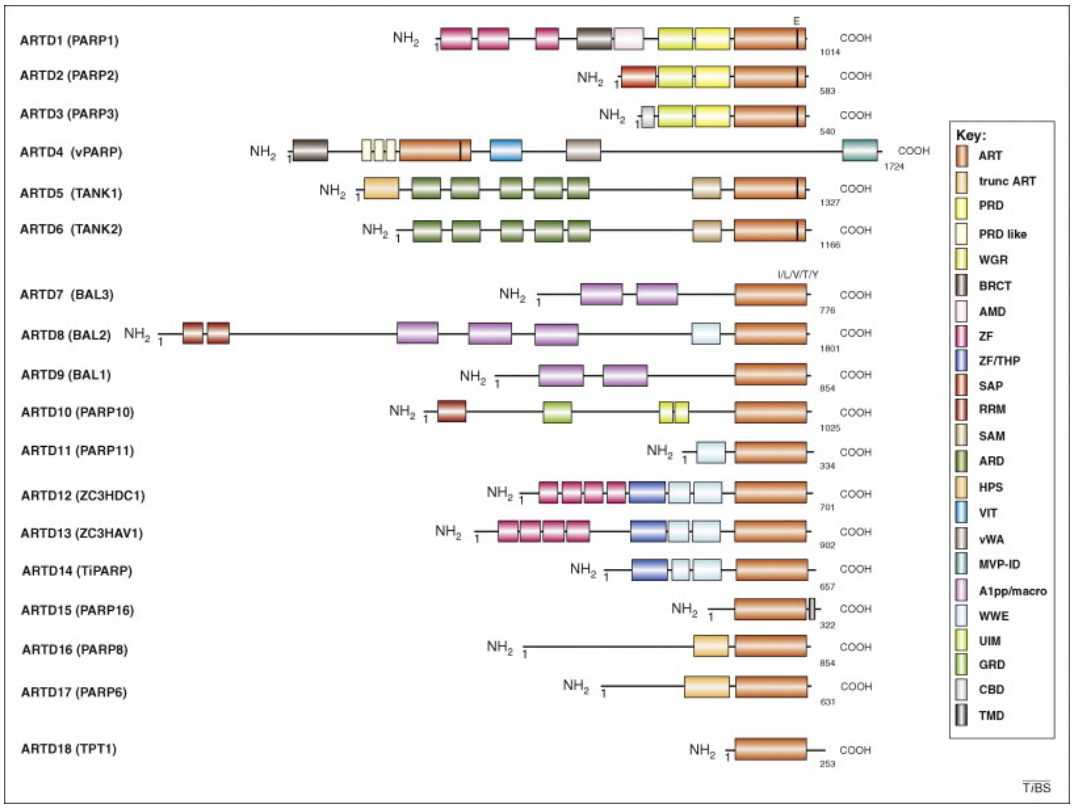

Fig.6 Domain architecture of the human ARTD (PARP) family.
The following BAL related functional domains are indicated: The ART domain is the catalytic core required for basal ART activity. The WWE domain is named after three conserved residues (W-W-E), and is predicted to mediate specific poly-ADP-ribose mediate protein-protein interactions in ubiquitin- and ADP-ribose conjugation systems. The Macro or A1pp domains are structurally related to the catalytic domain of enzymes that process ADP-ribose-1′-phosphate, a reaction product derived from ADP-ribose 1′-2′ cyclic phosphate generated by ARTD18/TpT. The Macro domain can also serve as mono-ADPr or O-acetyl-ADP-ribose binding module. ZF: zinc finger domains. RRM is a poly-ADP-ribose and RNA-binding/recognition motif. Within each ART domain, the region that is homologous to the ARTD signature (residues 859–908 of ARTD1) as well as the equivalent of the ARTD1 catalytic E988 is shaded. *(From Hottiger MO. et al., Toward a unified nomenclature for mammalian ADP-ribosyltransferases, Trends Biochem Sci., 2010 (135)*

2.3 Mono-ADP-ribose-protein hydrolases

The extent of post-translational modification by mono-ADP-ribosylation depends on mono-ADP-ribosyltransferases and mono-ADP-ribose-protein-hydrolases (mARHs) that antagonize each other ((138, 139) and reviewed in (126, 140)). Amino acid-mono-ADP-ribose-specific mARHs cleave the ribose-amino acid bond, leading to

release of mono-ADP-ribose and regeneration of the free reactive group of the corresponding amino acid residue ((138, 139) and reviewed in (126, 140)). This indicates that mono-ADP-ribosylation of proteins is a reversible process and that this modification is most likely of regulatory importance (126, 138, 140-143). Thus mARTs and mARHs are opposing arms of ADP-ribosylation cycles. The relevance of reversible mono-ADP-ribosylation of cytoplasmic proteins has obtained support from a recent study demonstrating that mono-ADP-ribose-arginine hydrolase-1 *(Marh1)* knockout mice are more sensitive to cholera toxin, Cholera toxin produced by *Vibrio cholerae* causes the devastating diarrhea of cholera by mono-ADP-ribosylation of α-subunits of intestinal heterotrimeric GTP-binding proteins, which results in severe water and electrolyte losses (144). Moreover, a recent study showed that *Marh1*(-/-) and *Marh1*(+/-) mice spontaneously develop lymphomas, adenocarcinomas, and metastases more frequently than wild-type mice (145). This study strongly indicates that tight control of protein mono-ADP-ribosylation levels mediated by ARH1 is essential for cancer suppression. However nothing is known about the ARTD specific mono-ADP-ribose-protein hydrolases that cleave the ribose-glutamic acid bond. Interestingly, recent studies showed that the human macro domain containing proteins MDO1, MDO2 and MDO3/c6orf130 could serve as bona fide O-acetyl-ADP-ribose (OAADP-ribose) hydrolases (146, 147), see also next section. Indeed, the proposed catalytic core structures of the macro domains of MDO1, MDO2 and MDO3/c6orf130 unexpectedly strongly resembles those of nucleotide triphosphate hydrolases and non-canonical poly-ADP-ribosyl-protein-glycohydrolases (148), strongly indicating that these macro domains may also function as an esterase towards E/D-ADP-ribose-ester bonds in ADP-ribosylated proteins, thus acting as ARTD specific mono-ADP-ribose-peptide-hydrolases.

2.4 Macro domains are ADP-ribose-protein modules

The macro domain is an evolutionary conserved protein module and is found associated with specific histone variants, such as macroH2A1.1, mono-ADP-ribosyltransferases, including BAL1/ARTD9, transcriptional coactivators such as ALC1/CHD1L and MDO1/LRP16 as well as with largely uncharacterized proteins, such as MDO2 and MDO3/c6orf130 (149, 150). In addition, a small number of animal RNA viruses encode macro domains (reviewed in (151)). (150). A subset of macro domains including those of mono-ADP-ribosyltransferases can serve as binding modules for free mono-ADP-ribose, OAADP-ribose and mono-ADP-ribosylated proteins (149, 150) and own unpublished observations). Remarkably, all BAL1-3/ARTD7-9 exist in different isoforms, containing either only one (BAL1/ARTD9 and BAL3/ARTD7) or 2 (BAL2/ARTD8) macro domains (own unpublished observations). Thus their proposed tumor promoting and immuno-modulatory activities could be regulated through the NAD/ADPr metabolite-binding activity of their macro domains. There is preliminary evidence, that macro domains may modulate transcription, for example they are able to repress transcription by binding to a promoter (11).

2.5 Roles of the B aggressive lymphoma proteins and ADP-ribosyltransferases BAL1/ARTD9, BAL2/ARTD8 and BAL3/ARTD7 in innate immunity and tumorigenesis

The macro domain-containing mono-ADP-ribosyltransferases and B-aggressive lymphoma proteins BAL1/ARTD9, BAL2/ARTD8 and BAL3/ARTD7 are nucleo-cytoplasmic shuttling proteins (25, 54, 55, 132, 152). Both, BAL2/ARTD8 and BAL3/ARTD7 are active mono-ADP-ribosyltransferases while no auto-modification or trans-mono-ADP-ribosyltransferase activity could be observed so far for BAL1/ARTD9 ((25, 127) and own unpublished observations). Thus BAL1/ARTD9 is most likely an inactive ARTD. All three BAL/ARTDs contain 2 or 3 macro domains in their N-terminal part (**Fig. 7**, see also section macro domains). BAL1-3/ARTD7-9 and BBAP/DTX3L are highly inducible proteins and only constitutively expressed in vivo at extremely low levels in lymphocyte-rich tissues (153). However, expression of BAL1-3/ARTD7-9 and BBAP/DTX3L is highly induced upon stimulation with IFNγ, IFNα/β, or upon infection with *H. pylori, S. typhimurium* and various RNA viruses ((153-157) and own unpublished observations).

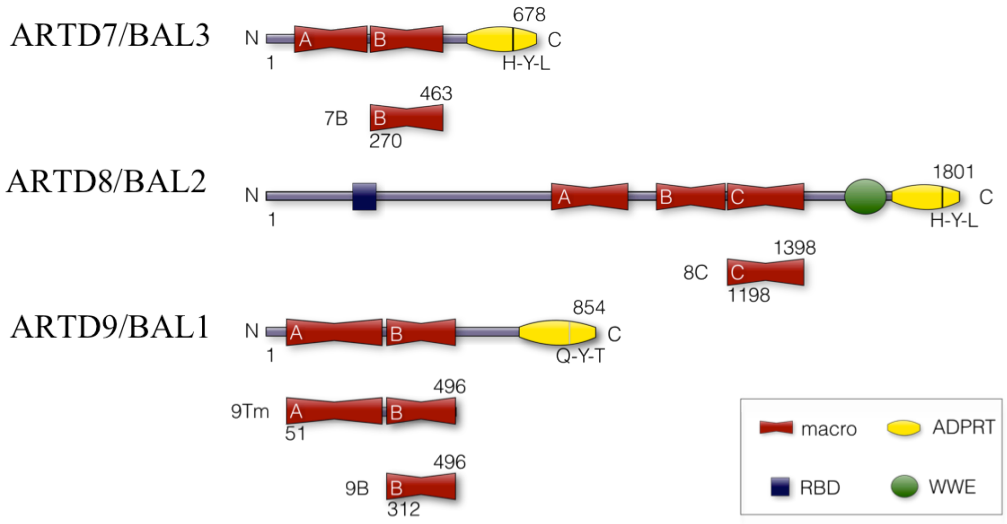

Fig.7 MacroARTD molecular structures.
(from Hans C. Winkler, Identification of p62 as interaction partner of ARTD9-is ARTD9 degraded by autophagy?Inaugural-Dissertation, 2012)

BAL1/ARTD9 has been initially identified as a novel risk-related gene that is constitutively over-expressed in aggressive chemo-resistant subsets of host response (HR) subtypes of diffuse large B-cell lymphoma (DLBCL) tumors (25, 55). In mice, BAL1/ARTD9 is developmentally regulated and mainly expressed (although at very low levels) in the thymus, in specific regions of the central nervous system and of the gut with the highest expression in the thymus and intestine (153). In adult mice, the highest levels of BAL1/ARTD9 expression (though still very low) were found in the medulla of the thymus and the white pulp of the spleen, suggesting a role for BAL1/ARTD9 in thymocytes maturation (153). In addition high expression levels of BAL1/ARTD9 were also detected in specific regions of the brain (dentate gyrus regions of the hippocampus) and in the epithelium of the duodenum, jejunum, ileum, and colon (153).

BAL1/ARTD9 has been proposed to be associated with lymphocytes migration and may promote the dissemination of malignant B cells in high-risk DLBCL in vivo (25). BAL1/ARTD9 over-expression has been shown to lead to increased responsiveness to the chemokine stromal cell-derived factor 1alpha (CXCL12/SDF-1) (25), though the molecular mechanism underlying this observation is not yet known. Doxo-cyclin-induced over.expression of BAL1/ARTD9 in BAL1/ARTD9 non-expressing low risk GCB-DLBCL led to the induction of a very small subset of interferon (IFN) related genes (25), thus providing preliminary evidence that BAL1/ARTD9 might play a direct role in IFN signaling pathways (25). However, the exact molecular functions and regulatory mechanisms of BAL1/ARTD9, especially in high-risk HR/ABC type DLBCLs, are not yet known and remains to be elucidated. For instance, it is not known whether any trans-mono-ADP-rbosylation activity is required for these functions.

Several recent studies by the Boothby lab provided preliminary evidence that BAL2/ARTD8, also referred to as collaborator of STAT6 (CoaSt6) interacts with STAT6 in vivo and amplifies STAT6-mediated gene expression pathways activated by interleukin 4 (132, 152). BAL2/ARTD8 has been suggested to functions as STAT6-specific co-regulator of IL-4 mediated gene expression in T-cells (132, 152). BAL2/ARTD8 is more weakly expressed than BAL1/ARTD9 in the thymus (153), but may also play a role during thymic development and function, because this organ seems to be the major site of BAL2/ARTD8 expression during development and in adulthood (153). In addition, expression of BAL2/ARTD8 seems also to be particularly high in the intestinal epithelium that covers Peyer's patches and colon lymphoid follicles (153). Remarkably, BAL2/ARTD8 functions as a highly selective co-activator/co-regulator of STAT-mediated gene expression, since it does not appear

to increase IFNγ-induced STAT1-dependent gene expression (132, 152). This study also provided preliminary evidence for a role of the BAL2/ARTD8-associated mono-ADP-ribosylation activity in STAT6-mediated transcriptional response (132). A catalytically fully inactive mutant of BAL2/ARTD8 was unable to enhance STAT6-mediated transcription of a test promoter (132). BAL2/ARTD8 has been suggested to mono-ADP-ribosylate p100, another co-activator of STAT6 (132). Interestingly, association of BAL2/ARTD8 with STAT6 was partially dependent on its macro domains (132, 152), indicating that STAT6 might be mono-ADP-rbosylated in vivo.

Recent studies provided first evidence that BAL2/ARTD8 may play crucial roles in vivo. A recent study using a mice model of BAL2/ARTD8 provided first evidence that BAL2/ARTD8 might be involved in mediating IL-4-induced proliferation and protection of B-cells against apoptosis following irradiation or growth factor withdrawal (158). In addition it has been suggested that BAL2/ARTD8 could influence Myc-induced oncogenesis by increasing the cellular metabolic rates (158). BAL2/ARTD8 knockout mice showed a reduced susceptibility to B cell lymphoma in a c-Myc-driven Burkitt lymphoma like tumor model (158). An earlier in vitro study already suggested that BAL2/ARTD8 might be connected with glycolysis, through the ubiquitinylation dependent regulation of phosphoglucose isomerase/autocrine motility factor activities (159). More recently, a study provided preliminary evidence that BAL2/ARTD8 can also act as an IL-4/STAT6 independent effector of the JNK2-dependent pro-survival signal in multiple myeloma (160). BAL2/ARTD8 is constitutively over-expressed in myeloma plasma and seems to be associated with disease progression and poor survival (160). BAL2/ARTD8 promotes the survival of myeloma cells by binding and inhibiting c-Jun N-terminal kinase (JNK)-1 kinase

activity (160). However, the exact molecular mechanisms and the functional role of its mono-ADP-rbosylation activity remain to be elucidated.

The third member, BAL3/ARTD7 exists in three different isoforms, BAL3/ARTD7 full-length, containing two macro domains and 2 short isoforms, containing only one macro domain ((54, 55) and own unpublished observations). BAL3/ARTD7 could therefore be regulated through the NAD metabolite-binding activity of its macro domains. BAL3/ARTD7 has been implicated in both regulates stress responses and microRNA activity in the cytoplasm as well as in macro domains mediated repression of transcription in the nucleus (54, 161, 162). BAL3/ARTD7 can act as transcriptional repressor when tethered to a promoter as a GAL4-DBD-fusion protein (54). Interestingly, this study provided evidence that the macro domain of BAL3/ARTD7 alone can repress transcription (54).

Collectively, the macro domain-containing mono-ADP-ribosyltransferases and B-aggressive lymphoma proteins BAL1/ARTD9 and BAL2/ARTD8 could act both as tumor promoting factors and regulators of innate immunity by modulating expression of host or pathogen induced tumor and pathogen-specific factors. Moreover, since BAL1/ARTD9 and BAL2/ARTD8 are involved in the regulation of gene expression activated by IFNγ and/or IL-4 respectively, they could antagonistically function in the immune response as well as in tumorigenesis (25, 132, 152). IFNγ and IL-4 can antagonize each other's function in thymocyte maturation and macrophage activation during the immune response (83-86).

2.6 Proposed Roles of the BAL1/ARTD9 binding partner and E3 ubiquitin ligase BBAP/DTX3L

BBAP/DTX3L was originally identified as a binding partner of BAL1/ARTD9 (163) and belongs to the E3 ligase family of Deltex (DTX) proteins (25). BBAP/DTX3L is also over-expressed in subtypes of high risk chemotherapy-resistant "host response" (HR) DLBCL (25, 163) and has been suggested to regulate the subcellular localization of BAL1/ARTD9 by a dynamic shuttling mechanism (25). BBAP/DTX3L has been suggested to facilitate nuclear export and shuttling of BAL1/ARTD9 from the nucleus to the cytoplasm (25). BBAP/DTX3L is essentially co-expressed with BAL1/ARTD9 during development and in adult tissues strongly indicating that BAL1/ARTD9 functions mainly in the cytoplasm in most cells. However, the exact molecular mechanism(s) remains to be elucidated. A recent study demonstrated that BBAP/DTX3L can selectively monoubiquitinate histone H4 lysine 91 and protects cells exposed to DNA damaging agents (164). However whether BBAP/DTX3L could also selectively monoubiquitinate signaling factors of the IFNγ or IFNα/β such as BAL1/ARTD9, STATs or IRF1 remain to be investigated.

2.7 Cross-talk between BAL1/ARTD9 and other ARTDs through ADP-ribose metabolites and (n)ADP-ribose binding modules?

Many ARTD family members contain domains, which could serve as mono and poly ADP ribose binding modules, such as RRM, WWE and macro domains (**Fig. 8**). Several recent studies provided biochemical and structural evidence that the macro domains of macro domain containing ARTDs can also serve as high-affinity binding modules for free ADP-ribose and mono-ADP-ribosylated proteins (165). Remarkably,

recent studies provided strong evidence that the WWE domain recognizes specifically poly(ADP-ribose) (PAR) but not mono-ADP-ribose by interacting with iso-ADP-ribose (166, 167). These studies further demonstrated that PAR binding is a common function for the WWE domain family including those of ARTDs (166, 167). Thus, the WWE domain containing ARTDs (such as BAL2/ARTD8) could specifically interact with poly-ADP-rbibosylated proteins including polymerizing ARTD members such as ARTD1. These studies strongly indicate that the macro domain containing ARTDs such as such as BAL1/ARTD9 or such as BAL2/ARTD8 may be not only regulated through OAADPR and free mono-ADPR but could also directly interact with each other or even with other mono-ADP-ribosylated and potentially also poly-ADP-ribosylated ARTDs, including the polymerizing ARTD member ARTD1.

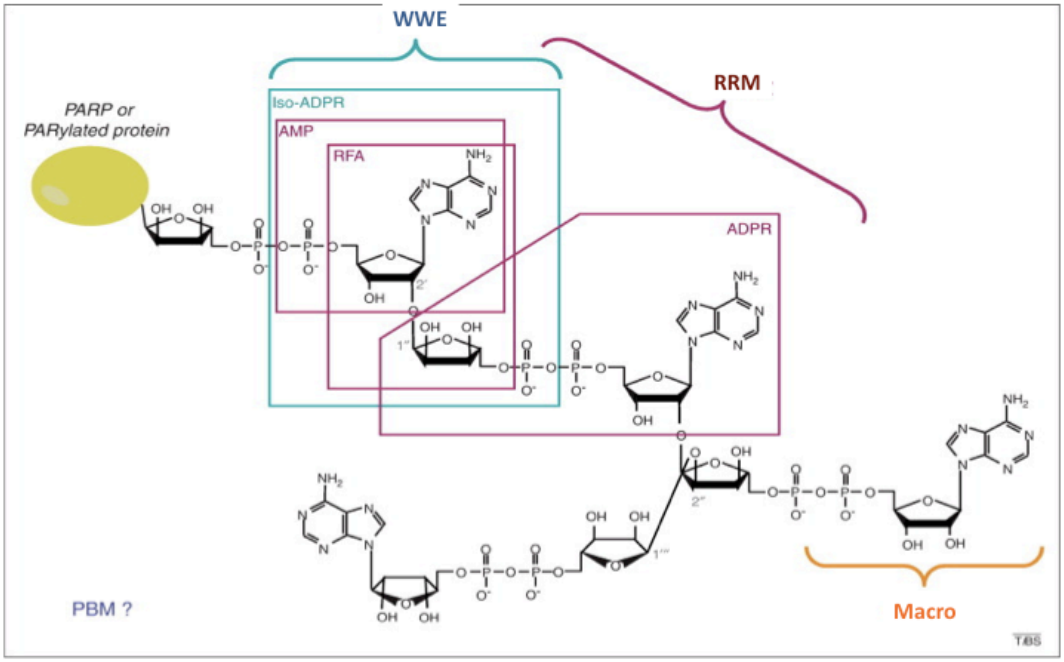

Fig.8 BAL related ARTD-associated (n)ADP-ribose binding modules
Macro-domain: mono-ADP-ribose binding domain, RRM: poly-ADP-ribose and RNA binding domain, WWE: poly-ADP-ribose binding domain. *(modified from Kalisch T. et al, New readers and interpretations of poly(ADP-ribosyl)ation TIBS 2012 (168)).*

2.8 ARTD/PARP inhibitors and concept of "synthetic/conditional lethality" effects towards tumors

Recent preclinical and clinical studies provided preliminary evidence that ARTD/PARP inhibitors might be promising drugs in the treatment of human malignancies including lymphomas and prostate cancer. Potent ARTD/PARP inhibitors with a high specificity towards ARTD1 and ARTD2, such as Olaparib (Astra Seneca/KUDOS) and ABT-888 (veliparib, Abbott Laboratories) have been developed that are not only effective as tumor cell radio- and chemo-sensitizers but as well as single agents to selective kill tumors in vivo. Biologically active doses of PARP inhibitors caused minimal syngenic toxicity in animal models and in phase I to II clinical trials. Several ARTD/PARP inhibitors are currently being further studied at the clinical trial phase III, specifically olaparib, and veliparib (http:/www.clinicaltrials.gov/). ARTD/PARP inhibitors are believed to exert their antitumor effects through at least two distinct but potentially complementary effects:

1) Through a "synthetic" lethal effect whereby continuous exposure of replicating cells to an ARTD/PARP inhibitor causes a significant increase in DNA DSB repair activity and defects in repair of DNA replication forks causing subsequent death in tumor cells deficient in HR. Two genes ('A' and 'B') are said to be 'synthetic lethal' if mutation of either gene alone is compatible with viability but simultaneous mutation of both genes causes death (45, 169-171). This concept can be extended to situations in which simultaneous mutation of two genes impairs cellular fitness more than mutation of either gene alone (45, 169-171). The concept of synthetic lethality is now used to choose anticancer drug targets. For instance, the protein products of

genes that are synthetic lethal to known cancer-causing mutations (such as ATM, BRCA1, BRCA2, and NBS1, if amenable to pharmacological attack (for example, if they encode an enzyme such as ARTD1), should theoretically represent excellent targets for anticancer therapy. Indeed, ARTD/PARP inhibitors seem to be highly effective as a single agent in patients whose tumors have germline or somatic defects in DNA damage repair genes (i.e. ATM, BRCA1, BRCA2, and NBS1) or defects in genes involved in phosphatase and tensin homolog gene (PTEN) signaling, confirming that inhibition of ARTDs (mainly ARTD1 and ARTD2) exerts "synthetic lethality" effect towards tumors [15]. ARTD1/PARP1 and its close relative ARTD2/PARP2 are known to play a role in the cellular response to DNA damage, and an important role for ARTD/PARP inhibitors as sensitizers to cytotoxic DNA damaging agents has emerged [12-14].

2) ARTD/PARP inhibitors also exert their antitumor effects through "conditional" lethality effect whereby ARTD/PARP inhibition may cause sensitization to therapeutic DNA damage in tumor cells. This "conditional" lethality approach exploits a postulated differential reliance on the DNA repair pathways for repair of therapeutic damage between malignant and normal tissues, such that ARTD/PARP inhibition might selectively enhance cytotoxicity from DNA damaging radiotherapy or chemotherapy.

However, the exact molecular mechanisms how ARTD/PARP inhibitors act and the specificity of many of these compounds are not yet known [15]. ARTD/PARP inhibitors have been initially thought to be highly specific towards ARTD1 and 2 (PARP1 and 2). A recent study evaluated a series of 185 small-molecule inhibitors, including research reagents and compounds being tested clinically, for the ability to bind to the catalytic domains of 13 of the 18 human ARTD family members (172).

These studies revealed that most of the best-known ARTD1 and ARTD2 inhibitors, including TIQ-A, 6(5H)-phenanthridinone, olaparib, ABT-888 and rucaparib, bind to several ARTD family members, including BAL2/ARTD8, BAL3/ARTD7 and ARTD10, strongly indicating that these molecules lack ARTD1/2 specificity and have promiscuous inhibitory activity (172).

The lethal effects of ARTD/PARP inhibitors are mainly investigated in DNA repair deficient tumors such as triple-negative breast carcinomas, androgen-insensitive prostate tumors, BRCA-deficient or triple negative ovarian cancer or colon cancer often in combination with other DNA damaging chemotherapeutic agents (173-176). Only few is known whether PARP inhibitors could be also used to target other types tumors such as DLBCL. For instance, recent studies provided preliminary evidence that ARTD/PARP inhibitors can lead to an increase in cytotoxicity in mantle cell lymphoma harboring mutations in both ATM and p53 (177-179).

3. AIM OF THE THESIS

The mayor aim of this project is to elucidate the functional roles of the intracellular mono-ADP-ribosyltransferase and B-aggressive lymphoma protein BAL1/ARTD9 in high-risk diffuse large B-cell lymphoma associated with constitutive IFN/STAT1 signaling and to investigate whether BAL1/ARTD9 could (co)-regulate the expression of IFNγ-dependent tumor suppressors and oncogenes in high-risk diffuse large B-cell lymphoma and mediate survival in diffuse large B-cell lymphoma. Thus identify new (lethal) regulatory connections between STAT signaling pathways and mono-ADP-ribosylation as a potential novel therapeutic target in prostate cancer. The project addresses 4 different tasks:

Task1: Does BAL1/ARTD9 regulate IFN/STAT dependent signaling in HR-DLBCL ?:

Task2: Does BAL1/ARTD9 act as corepressor or coactivator of IFN/STAT1 signaling in DLBCL?

Task3: Are the macrodomains required for the activity of BAL1/ARTD9 in DLBCL?

Task4: Does BAL1/ARTD9 mediates proliferation and survival in DLBC?

Working Hypothesis:

BAL1/ARTD9 negatively regulates IFNγ/STAT1-dependent tumor suppressors such as IRF1, while positively regulating proto-oncogene products such IRF2 and as a consequence mediates survival in high-risk diffuse large B-cell lymphoma.

4. RESULTS

4.1 Original research publications

BAL1/ARTD9 represses the anti-proliferative and pro-apoptotic IFNγ-STAT1-IRF1-53 axes in diffuse large B-cell lymphoma

Rosalba Camicia,[1] Samia B. Bachmann,[1] Hans C. Winkler,[1] Marc Beer,[2] Marianne Tinguely[2] Eugenia Haralambieva [2] and Paul O. Hassa[1] (JCS, March 2013)

Running title: BAL1 inhibits IRF1

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BAL1/ARTD9 represses the anti-proliferative and pro-apoptotic IFNγ–STAT1–IRF1–p53 axis in diffuse large B-cell lymphoma

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Summary

The B-aggressive lymphoma-1 protein and ADP-ribosyltransferase BAL1/ARTD9 has been recently identified as a risk-related gene product in aggressive diffuse large B-cell lymphoma (DLBCL). BAL1 is constitutively expressed in a subset of high-risk DLBCLs with an active host inflammatory response and has been suggested to be associated with interferon-related gene expression. Here we identify BAL1 as a novel oncogenic survival factor in DLBCL and show that constitutive overexpression of BAL1 in DLBCL tightly associates with intrinsic interferon-gamma (IFNγ) signaling and constitutive activity of signal transducer and activator of transcription (STAT)-1. Remarkably, BAL1 stimulates the phosphorylation of both STAT1 isoforms, STAT1α and STAT1β, on Y701 and thereby promotes the nuclear accumulation of the antagonistically acting and transcriptionally repressive isoform STAT1β. Moreover, BAL1 physically interacts with both STAT1α and STAT1β through its macrodomains in an ADP-ribosylation-dependent manner. BAL1 directly inhibits, together with STAT1β, the expression of tumor suppressor and interferon response factor (IRF)-1. Conversely, BAL1 enhances the expression of the proto-oncogenes IRF2 and B-cell CLL/lymphoma (BCL)-6 in DLBCL. Our results show for the first time that BAL1 represses the anti-proliferative and pro-apoptotic IFNγ–STAT1–IRF1–p53 axes and mediates proliferation, survival and chemo-resistance in DLBCL. As a consequence constitutive IFNγ–STAT1 signaling does not lead to apoptosis but rather to chemo-resistance in DLBCL overexpressing BAL1. Our results suggest that BAL1 may induce an switch in STAT1 from a tumor suppressor to an oncogene in high-risk DLBCL.

Key words: BAL1/ARTD9, Diffuse large B-cell lymphoma, Macrodomains, IFNγ-STAT1-signaling, Survival, ADP-ribosylation, IRF1, p53

Introduction

The B-aggressive lymphoma-1 protein and ADP-ribosyltransferase BAL1/ARTD9, here referred to as BAL1, is a nucleocytoplasmic shuttling protein that has been identified as a potential risk-related gene product in diffuse large B-cell lymphoma (DLBCL) (Aguiar et al., 2000; Juszczynski et al., 2006). BAL1 belongs to the diphtheria-toxin-related ADP-ribosyltransferase (ARTD) family (former PARP) of intracellular mono- and poly-ADP-ribosyltransferases (Aguiar et al., 2005; Aguiar et al., 2000; Hottiger et al., 2010). No auto-modification or mono-ADP-ribosyltransferase activity has been observed for BAL1 so far (Aguiar et al., 2005). BAL1 contains two evolutionarily conserved macrodomains. Macrodomains have been recently shown to act as a binding module for free and protein-linked mono- or poly-ADP-ribose (Moyle and Muir, 2010; Timinszky et al., 2009). BAL1 is constitutively expressed in a subset of aggressive chemo-resistant high-risk subtypes of DLBCL, which are associated with an active but ineffective IFNγ-mediated host inflammatory response (HR) (Aguiar et al., 2000; Juszczynski et al., 2006). BAL1 has been suggested to be involved in lymphocyte migration and modulation of IFN-signaling-related gene expression in DLBCL (Juszczynski et al., 2006). However, the exact molecular functions of endogenous BAL1 and its regulatory mechanisms in aggressive DLBCL have not been investigated and remain to be elucidated.

DLBCL is a clinically heterogeneous lymphoid malignancy and the most common subtype of non-Hodgkin's lymphoma in adults, with one of the highest mortality rates (Shaffer et al., 2012). DLBCL has been subdivided into distinct classes (Rosenwald et al., 2002; Shipp et al., 2002). Recently, a high-risk subclass with worse clinical outcomes that is associated with an active but ineffective host inflammatory response has been identified (Monti et al., 2005), known as HR-DLBCL. HR-DLBCL is associated with increased expression of inflammatory mediators and downstream targets of interferon gamma (IFNγ) signaling (Monti et al., 2005). HR-DLBCL lacks most of the common cytogenetic abnormalities and the exact mechanisms of transformation in these tumors remain to be elucidated (Abramson and Shipp, 2005; Monti et al., 2005). The clinical outcome of the HR-DLBCL cluster is not improved, despite the increased inflammatory response (Abramson and Shipp, 2005; Monti et al., 2005). Thus, it has been suggested that either the host immune responses are inhibited by counter-regulatory mechanisms or HR-DLBCL tumors are resistant to chemotherapy, or a combination of both (Abramson and Shipp, 2005; Monti et al., 2005).

IFNγ exhibits both pro- and anti-tumor properties, depending on the context and cancer type (Dunn et al., 2006). Initially, IFNγ helps protect the host from tumor formation and development (immunosurveillance), but subsequently IFNγ can also promote the tumors to resist the attack (immunoediting) (Dunn et al., 2006; Juszczyński et al., 2008; Lukacher, 2002). The anti-tumor activity of IFNγ is mediated through the signal transducer and activator of transcription 1 (STAT1) and interferon response factor 1 (IRF1) (Taniguchi et al., 2001). Many tumors lack IRF1 or have reduced IRF1 expression levels (Green et al., 1999). Both STAT1 and its mayor target gene *IRF1* have been shown to positively modulate p53-activated apoptotic pathways (Taniguchi et al., 2001; Townsend et al., 2004). Remarkably, recent studies provided evidence that *STAT1* can also act as a proto-oncogene in solid cancers (Khodarev et al., 2004). However, the exact molecular mechanisms of how STAT1 acts as an oncogene are not yet known.

We have identified BAL1 as a novel co-repressor for the transcriptional repression of tumor suppressor IRF1 and a co-activator for the transcriptional activation of the proto-oncogenes *IRF2* and B-cell CLL/lymphoma 6 (*BCL6*). BAL1 interacts with the IFNγ receptor (IFNGR) complex and enhances tyrosine phosphorylation of both isoforms of STAT1 on Y701 and their subsequent nuclear translocation, thereby promoting the nuclear accumulation of the antagonistically acting and transcriptionally repressive isoform STAT1β. BAL1 interacts with both STAT1 isoforms through its macrodomains in an ADP-ribosylation-dependent manner, and together with STAT1β inhibits the *IRF1* promoter. BAL1 counteracts the IFNγ-dependent anti-proliferative and pro-apoptotic IFNγ–STAT1–IRF1–p53 axes and as a consequence, mediates proliferation, chemo-resistance and survival in high-risk DLBCL.

Results

BAL1 is constitutively expressed in DLBCL and is associated with aberrant IFNγ/STAT1 signaling

Previous reports showed that BAL1 is constitutively overexpressed in high-risk primary DLBCL with an active host inflammatory response (Aguiar et al., 2005; Aguiar et al., 2000; Juszczynski et al., 2006). Overexpression of BAL1 has also been observed in the aggressive ABC-DLBCL cell lines OCI-Ly3 and OCI-Ly10 as well as in the GCB-HR-like DLBCL cell line SUDHL7 (Aguiar et al., 2005; Aguiar et al., 2000; Juszczynski et al., 2006). IFNγ/STAT1–IRF1 signaling has been shown to stimulate the expression of BAL1 *in vitro* (Juszczynski et al., 2006; Shi et al., 2011). IFNγ signaling is mediated through activation of the IFNγ receptor and Janus kinases (JAK) 1 and 2 that lead to tyrosine phosphorylation of STAT1 on Y701, homodimerization and translocation of STAT1 to the nucleus where it induces the transcription of IFNγ-stimulated genes such as *IRF1* (Dunn et al., 2006). In order to confirm these data and to investigate whether constitutive expression of endogenous BAL1 is associated with constitutively active STAT1 signaling and IRF1 expression we analyzed STAT1 activity in different BAL1-expressing and -non-expressing DLBCL cell lines including the chemo-resistant GCB-related HR-DLBCL cell line SUDHL7.

Indeed, our immunoblot analysis of BAL1, STAT1, pSTAT1, STAT2, pSTAT2 and IRF1 expression, revealed that constitutively expressed BAL1 is tightly associated with intrinsic IFNγ signaling and constitutively active STAT1 (Fig. 1A,B). Moreover, our STAT1-knockdown analysis using siRNA revealed that constitutive expression of BAL1 is strictly dependent on the transcriptional activity of STAT1 (Fig. 1C). Subsequent chromatin immunoprecipitation analysis revealed that endogenous STAT1 is recruited to the *BAL1* promoter (Fig. 1D), strongly indicating that STAT1 directly activates *BAL1* gene expression. STAT2 activity appears to be normal in all DLBCL cell lines tested and not involved in upregulation of BAL1 in HR-DLBCL cells, demonstrated by the absence of phosphorylated STAT2. The observed IFNγ-induced BAL1 expression tightly correlates with the induced activity of STAT1 in the ABC-DLBCL cell lines OCI-Ly3 and OCI-Ly10. However, the lower expression levels of BAL1 in OCI-Ly3 and OCI-Ly10 could also be mediated through the constitutively high activity of NF-κB family members in these ABC DLBCL cell lines (Davis et al., 2010; Ngo et al., 2011; Shaffer Iii et al., 2012). Surprisingly, tumor suppressor IRF1, a major target of STAT1 (Taniguchi et al., 2001), is strongly downregulated in the presence of both constitutively and inducibly expressed BAL1, whereas it is upregulated in the absence of constitutively expressed BAL1 (Fig. 1B). These observations indicate that BAL1 might act as a transcriptional repressor of the *IRF1* gene.

Interestingly, our analysis of primary DLBCL tumors revealed that 18% of the primary tumor samples analyzed showed positive nuclear staining for all three analyzed markers IRF2, STAT1-pY701 and STAT1-pS727, whereas benign tissue did not show this pattern (Fig. 1E; supplementary material Fig. S1D,E).

BAL1 inhibits tumor suppressor IRF1 and activates oncogenic BCL6 to mediate proliferation

In order to test whether BAL1 inhibits the tumor-suppressing IFNγ–STAT1–IRF1 axis and thus directly stimulates cell proliferation, we first analyzed the proliferation of SUDHL7 cells in which BAL1 had been knocked down (SUDHL7-shBAL1) and control cells (SUDHL7-shmock). Remarkably, this experiment revealed that knockdown of BAL1 strongly inhibits proliferation (Fig. 2A; supplementary material Fig. S2A). We next investigated whether BAL1 is directly required for the transcriptional downregulation of tumor suppressor genes and upregulation of proto-oncogenes involved in proliferation and survival in DLBCL. We first analyzed the expression levels of the IFNγ/STAT1-dependent tumor suppressors and oncogene products such as STAT1, IRF1 and IRF2. Our results demonstrate that the expression of tumor suppressor IRF1 is strongly upregulated in BAL1-knockdown cells, whereas the expression level of the proto-oncogene IRF2 is strongly reduced (Fig. 2B,C). In contrast, the expression of STAT1 and its isoforms is not regulated by BAL1 in SUDHL7 (Fig. 2C; supplementary material Fig. S2B). STAT1 exists in two mayor isoforms, the full-length isoform STAT1α, which mainly acts as a sequence-specific activator of gene expression and STAT1β, lacking a complete transactivation domain and acting as a transcriptional repressor and antagonist of STAT1α (Baran-Marszak et al., 2004; Zakharova et al., 2003). Remarkably, our expression analysis demonstrates that BAL1 also stimulates the expression of another IFNγ/STAT1-independent crucial proto-oncogene *BCL6*, while concomitantly repressing the tumor-suppressor PR-domain-containing 1 (*Prdm1*) gene, the gene product of which is the B-lymphocyte-induced-maturation protein-1 (BLIMP1), an antagonist of BCL6 (Fig. 2B,C). The observed transcriptional upregulation of *BCL6* by BAL1 is also consistent with previous reports demonstrating that subsets of high-risk DLBCL are dependent on BCL6 (Saito et al., 2009).

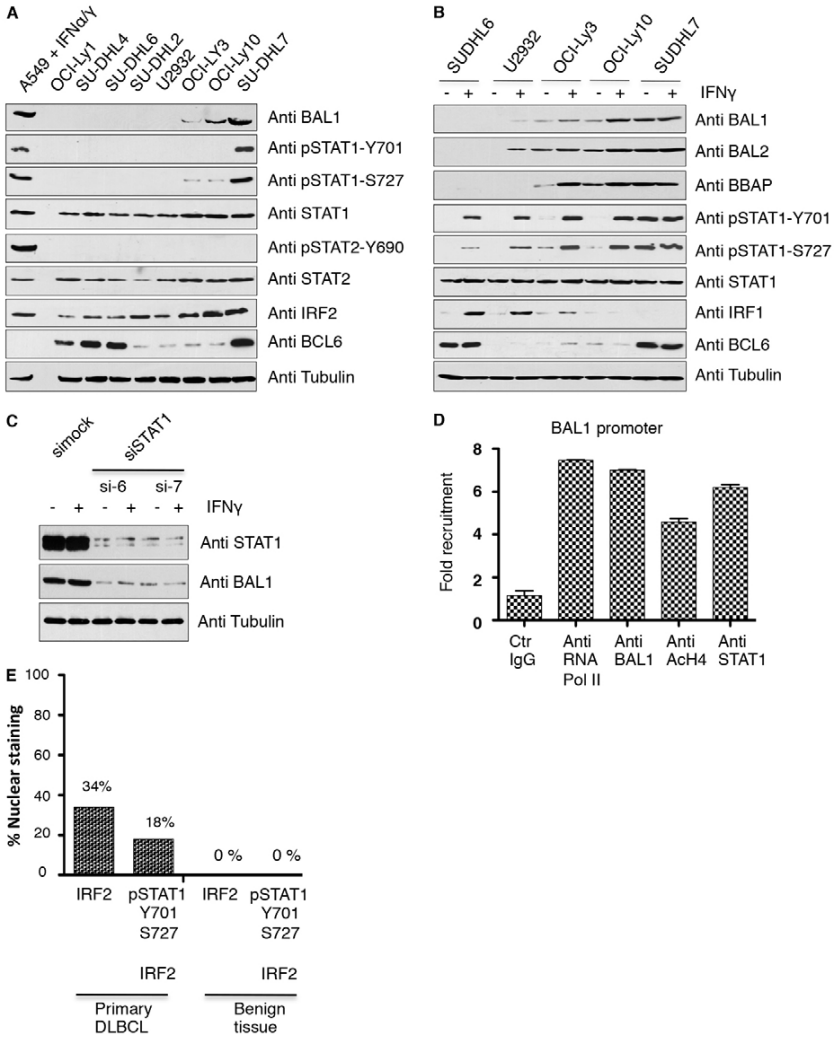

Fig. 1. BAL1 is constitutively expressed in DLBCL associated with constitutively active STAT1 signaling. (**A**) Immunoblot analysis of untreated GCB-DLBCL cell lines (OCI-Ly1, SUDHL4 and SUDHL6), ABC-DLBCL cell lines (SUDHL2, U2932, OCI-Ly3 and OCI-Ly10) and GCB-HR-DLBCL cell line SUDHL7. Whole-cell extracts were separated by SDS-PAGE, blotted and subsequently probed with antibodies for STAT1, pSTAT1(Y701), pSTAT1(S727), STAT2, pSTAT2(Y690), BAL1, IRF2, BCL6 and tubulin. (**B**) Immunoblot analysis of STAT1 signaling in GCB-, ABC- and HR-GCB-DLBCL cell lines untreated or treated with 1000 U/ml IFNγ for 8 hours. GCB-DLBCL cell line (SUDHL6), ABC-DLBCL cell lines (U2932, OCI-Ly3 and OCI-Ly10) and the GCB-HR-DLBCL cell line SUDHL7 were untreated or treated with IFNγ for 8 hours and then whole-cell extracts separated by SDS-PAGE and subsequently probed with antibodies for BAL1, BAL2/ARTD8, BBAP/DTX3L STAT1, pSTAT1(Y701), pSTAT1(S727), IRF1, BCL6 and tubulin. (**C**) Immunoblot analysis of BAL1 and STAT1 expression using whole-cell extracts of SUDHL7-simock and siSTAT1-knockdown cells untreated or treated with 1000 U/ml IFNγ for 8 hours. (**D**) Chromatin immunoprecipitation (ChIP) analysis of the *BAL1* promoter for H4K16-acetylation, STAT1, BAL1 and RNA-Pol-II recruitment in SUDHL7 cells, using anti-STAT1, anti-BAL1, anti RNA-Pol-II, anti-H4K16Ac and control (Ctr) antibodies. (**E**) Analysis of primary DLBCL tumors: the bars indicate the percentage of positive nuclear staining for IRF2 alone and for all three analyzed markers IRF2, pSTAT1(Y701) and pSTAT1(S727) in primary DLBCL tumors and benign samples. For a detailed description of the scoring system see Materials and Methods and supplementary material Tables S3, S4.

BCL6 is overexpressed in the majority of patients with aggressive DLBCL (Saito et al., 2009). Constitutive expression of BCL6 mediates lymphomagenesis through aberrant proliferation and cell survival (Saito et al., 2009). Remarkably, BAL1 also enhances the expression of BAL2/ARTD8 and the BAL2/ARTD8 target, the oncogenic PIM1 kinase (Cho et al., 2009a) (Fig. 2B). BAL2/ARTD8 is another macrodomain-containing ARTD family member (Cho et al., 2009b; Goenka and Boothby, 2006; Goenka et al., 2007).

Next we tested whether BAL1 directly regulates *BCL6* and *IRF1* gene expression. Indeed chromatin immunoprecipitation analysis of SUDHL7 cells revealed that endogenous BAL1 is recruited to both the STAT1-dependent *IRF1* promoter and to the STAT1-independent *BCL6* promoter (Fig. 2D), strongly indicating that BAL1 directly repress *IRF1* gene expression and stimulates *BCL6* gene expression on the level of transcriptional activation (Fig. 2D). Activation of BCL6 tightly correlates with H4K16-acetylation and RNA-PolII recruitment, whereas repression of IRF1 correlates with absence of H4K16 acetylation and RNA-PolII recruitment (Fig. 2D). In contrast, activation of PIM1 is not directly mediated by *BCL1* (supplementary material Fig. S2D). As expected, STAT1 is not required for the recruitment of BAL1 to the *BCL6* promoter.

BAL1 inhibits the IRF1-mediated cell death and activates BCL6-mediated survival

In order to test whether the observed stimulation of BCL6 expression and repression of IRF1 by BAL1 also has direct effects on cell survival we treated SUDHL7 BAL1-knockdown and sh-mock control cells with etoposide and/or doxorubicin and subsequently analyzed the survival and proliferation as well as gene expression (Fig. 3A–C). Remarkably, these experiments revealed that BAL1 not only blocks IFNγ–STAT1–IRF1-mediated apoptosis and inhibition of growth, but also reverses the chemo-resistance of SUDHL7 (Fig. 3A,B). Absence of BAL1 strongly increases the expression of pro-apoptotic and anti-proliferative gene products including p21, BAD, p53 or CASP3 while simultaneously downregulating the expression of pro-survival gene products such BCL2 or BCL-XL (Fig. 3B). IRF1 has been shown to induce both ligand-dependent (extrinsic) and ligand-independent (intrinsic) caspase-mediated apoptosis (Stang et al., 2007). Recent studies demonstrated that IRF1 inhibits the expression of pro-survival members of the BCL2 family and induces the expression and activation of pro-apoptotic and anti-proliferative gene products, including p53, p21 and CASP3

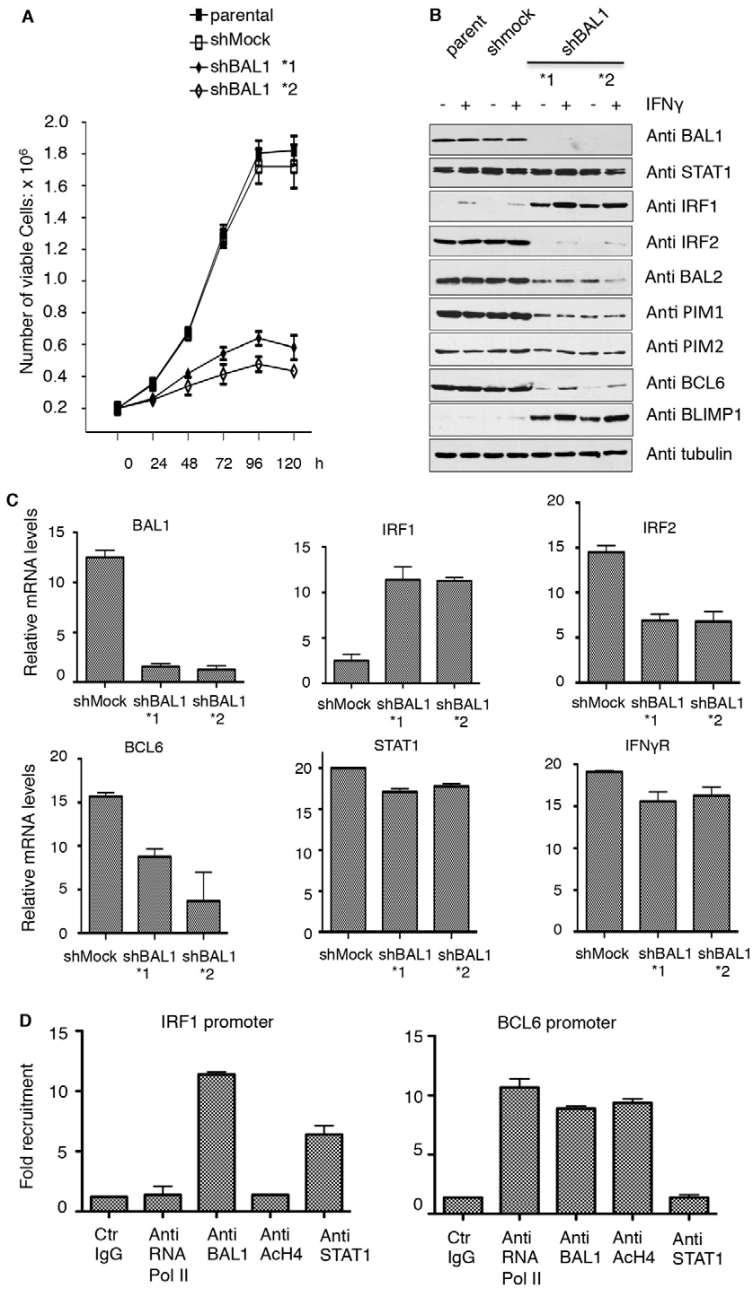

Fig. 2. BAL1 inhibits IRF1 and activates BCL6 to mediate proliferation in DLBCL. (**A**) Cell proliferation analysis of SUDHL7-parental, stable SUDHL7-shmock and BAL1-knockdown cells over 120 hours, was assessed by a Trypan Blue exclusion assay. Cells were seeded at 0.2×10^6 cells/ml in triplicate in six-well dishes and counted every day for 5 days. Values are means ± s.d. from three independent experiments performed in triplicate. (**B**) Immunoblot analysis of tumor suppressor gene and proto-oncogene products. Parental wild-type SUDHL7, shmock-RNA and *BCL1*-shRNA knockdown cells were untreated or treated with 1000 U/ml IFNγ for 8 hours and then whole-cell extracts separated by SDS-PAGE, blotted and subsequently probed with antibodies for BAL1, BAL2, STAT1, IRF1, IRF2, BCL6, BLIMP1, PIM1, PIM2 and tubulin. (**C**) Gene expression analysis of stable SUDHL7 shmock RNA and *BCL1*-shRNA knockdown cells. mRNAs were isolated from transient SUDHL7 shRNA knockdown cells and *BAL1*, *STAT1*, *IRF1*, *IRF2*, *IFNGR2* and *BCL6* mRNA levels were measured by qPCR and normalized against *GAPDH*. (**D**) ChIP analysis of *IRF1* and *BCL6* promoters for H4K16-acetylation, STAT1, BAL1 and RNA-Pol-II recruitment in SUDHL7 cells using anti-STAT1, anti-BAL1, anti RNA-Pol-II, anti-H4K16Ac and control (Ctr) antibodies.

(Choo et al., 2006; Schwartz et al., 2011; Shi et al., 2011). Conversely, it has been previously demonstrated that BCL6 can also suppress both the basal and the induced expression levels of tumor-suppressor genes *PRDM1/BLIMP1* and *p53*, the cyclin-dependent kinase inhibitor gene *p21* as well as the anti-apoptotic proto-oncogene *BCL2* in DLBCL (Phan and Dalla-Favera, 2004; Saito et al., 2009). Interestingly, phosphorylation and inactivation of the pro-apoptotic protein BAD is strongly reduced in absence of BAL1 (Fig. 3B). However, phosphorylation of BAD at S112 is probably not directly regulated by BAL1 but rather through BAL2 and its target, the oncogenic kinase PIM1. Several studies showed that phosphorylation of BAD on serine 112 is also mediated by PIM1 and contributes to cell survival in B-cell lymphoma (Aho et al., 2004; Chen et al., 2008; Yan et al., 2003).

The observed concomitant overexpression of BCL2 and BCL6 in SUDHL7 cells also indicates that BAL1 blocks BCL6-mediated repression of the *BCL2* gene, which is frequently disrupted in DLBCL (Saito et al., 2009). We next investigated whether the siRNA-mediated knockdown of STAT1 could inhibit the pro-apoptotic and/or anti-proliferative pathways in absence of BAL1 and thus could rescue proliferation and/or survival in these cells. These experiments revealed that siRNA-mediated knockdown of STAT1 in SUDHL7 cells indeed blocks the IRF1-mediated pro-apoptotic and anti-proliferative pathways (Fig. 3D–F). However,

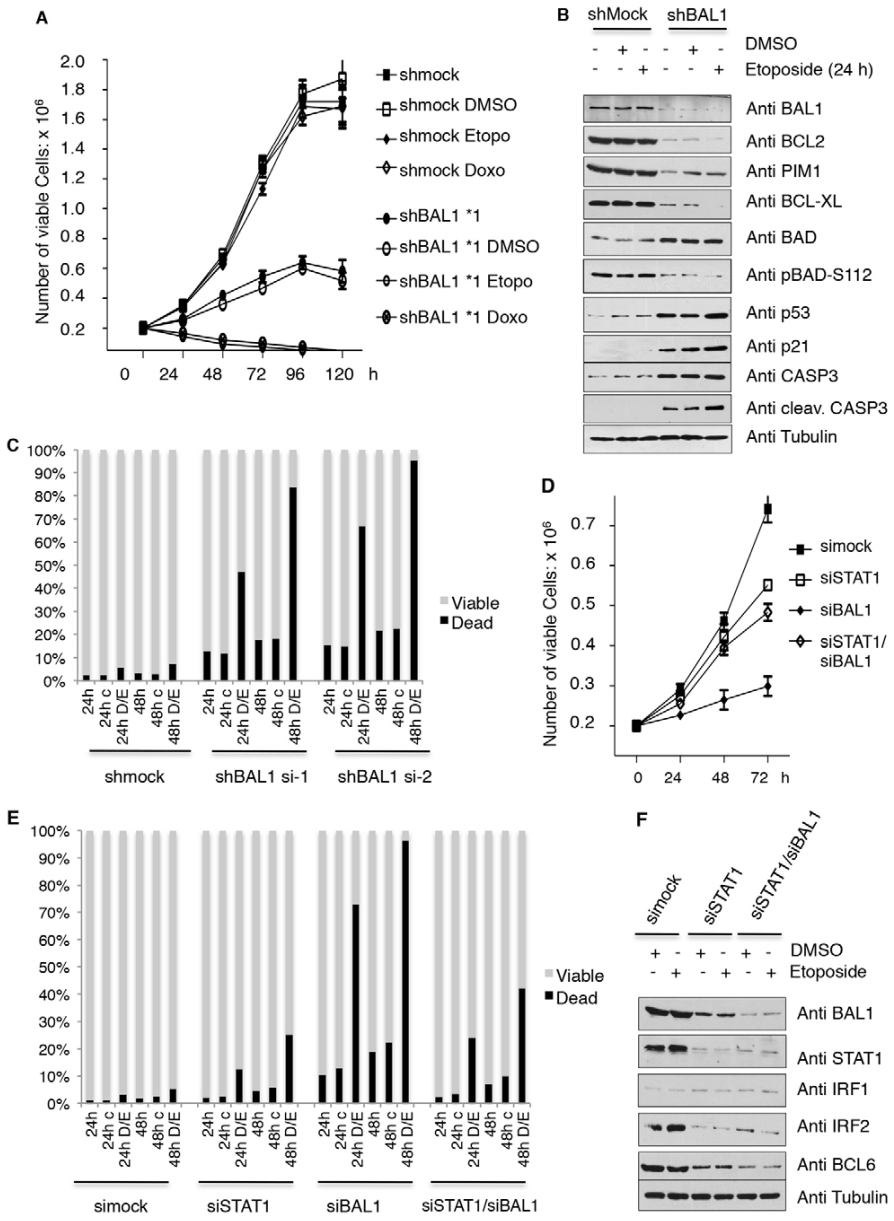

Fig. 3. BAL1 inhibits the IRF1-mediated pro-apoptotic pathways and activates the BCL6-mediated survival pathways. (**A**) Cell proliferation analysis of parental SUDHL7 cells, stable SUDHL7 shmock and BAL1-knockdown cells treated with etoposide (Etopo; 25 μM) and/or doxorubicin (Doxo; 5 μM) was assessed by a Trypan Blue exclusion assay. Cells were seeded at 0.2×10^6 cells/ml in triplicate in six-well dishes, treated as indicated and counted every day for 5 days. Values are means ±s.d. from three independent experiments performed in triplicate. (**B**) Immunoblot analysis of pro-apoptotic and survival factors. SUDHL7 shmock-RNA and BAL1-shRNA knockdown cells were untreated or treated with Etoposide (25 μM) for 24 hours and then whole-cell extracts separated by SDS-PAGE, blotted and subsequently probed with antibodies for BAL1, BCL2, BCL-xL, PIM1, BAD, pBAD-S112, p53, p21, Casp3, cleaved Casp3 and tubulin. (**C**) Cell viability analysis of parental SUDHL7 cells, stable SUDHL7 shmock and BAL1-knockdown cells untreated (control, c) treated with etoposide (E; 25 μM) and/or doxorubicin (D; 5 μM) was assessed by a Trypan Blue exclusion assay. Cells were seeded at 0.2×10^6 cells/ml in triplicate in six-well dishes, treated as indicated and counted every day for 5 days. Values are mean percentages from three independent experiments performed in triplicate. (**D**) Cell proliferation analysis of untreated SUDHL7-si mock, siBAL1, siSTAT1 and siSTAT1/BAL1 knockdown cells. (**E**) Cell viability analysis of SUDHL7-simock, siBAL1, siSTAT1 and siBAL1/siSTAT1-knockdown cells untreated (control, c) or treated with etoposide (25 μM) and doxorubicin (5 μM) was assessed by a Trypan Blue exclusion assay. Cells were seeded at 0.2×10^6 cells/ml in triplicate in six-well dishes, treated as indicated and counted every day for 5 days. Values are means from three independent experiments performed in triplicate. (**F**) Immunoblot analysis of siSTAT1 knockdown cells. SUDHL7 simock-RNA, STAT1-siRNA and STAT1-siRNA/BAL1-siRNA knockdown cells were untreated or treated with Etoposide (25 μM) for 24 hours and then whole cell extracts separated by SDS-PAGE, blotted and subsequently probed with antibodies for STAT1, BAL1, IRF1, IRF2 and BCL6.

the observed proliferation defects and increased cell death in siBAL1 cells could not be fully rescued in presence of siSTAT1 RNA, indicating first that STAT1 itself is required for survival and proliferation, most probably through IRF2, and second, the presence of overexpressed BCL6 is equally important for survival in this cell line (Fig. 3D–F).

BAL1 forms complexes with STAT1α and STAT1β through its macrodomains and is recruited by STAT1 to its target promoters

Since tumor suppressor IRF1 is a major target of STAT1 we investigated whether BAL1 could form a complex with STAT1 or other IFN-related STAT members *in vivo*. We first performed co-immunoprecipitation experiments with HA-tagged BAL1 and FLAG-tagged STAT1-6 transiently coexpressed in HEK293 cells, as well as with endogenously expressed BAL1 and STAT1 in SUDHL7 cells. Our interaction studies revealed that BAL1 specifically interacts with both STAT1 and STAT2 (Fig. 4A), indicating that endogenous BAL1 forms complexes with both IFNγ-induced STAT1 homodimers (Fig. 4B). Moreover, BAL1 interacts with both STAT1α and STAT1β isoforms (Fig. 4C). No interaction between BAL1 and other STATs (STAT3–6) could be observed under the tested conditions (Fig. 4A). We next tested which domain of BAL1 is required for the observed interaction by performing co-immunoprecipitation experiments with HA-tagged full-length BAL1 and deletion mutants coexpressed together with FLAG-tagged STAT1 or STAT2 in HEK293 cells. Remarkably, these mapping

experiments revealed that the observed interactions between BAL1 with STAT1 are mediated through both macrodomains (Fig. 4D; supplementary material Fig. S3A) and thus might be dependent on n-ADP-ribosylation. Subsequent co-immunoprecipitation experiments revealed that the interaction is indeed mediated by (mono)-ADP-ribosylation (Fig. 4E). Thus, our results suggest a potential regulatory connection between ADP-ribose binding modules and mono-ADP-ribosylation-dependent signaling and gene expression in high-risk DLBCL and other B-cell lymphomas. STAT1 is modified *in vitro* by BAL2/ARTD8 and ARTD10 (data not shown). However, we were unable to elucidate the exact mechanisms *in vivo* and thus we have no evidence so far that STATs are ADP-ribosylated *in vivo*. Analyzing protein mono-ADP-ribosylation *in vivo* is a difficult task because antibodies specifically recognizing mono-ADP-ribosylated glutamate or arginine residues are lacking and ADP-ribosylation of proteins *in vivo* is not easily analyzed by mass spectrometry (Hottiger et al., 2010). Moreover, there are no ARTD-family-member-specific ARTD/PARP inhibitors available that would specifically target BAL2, BAL3 or other

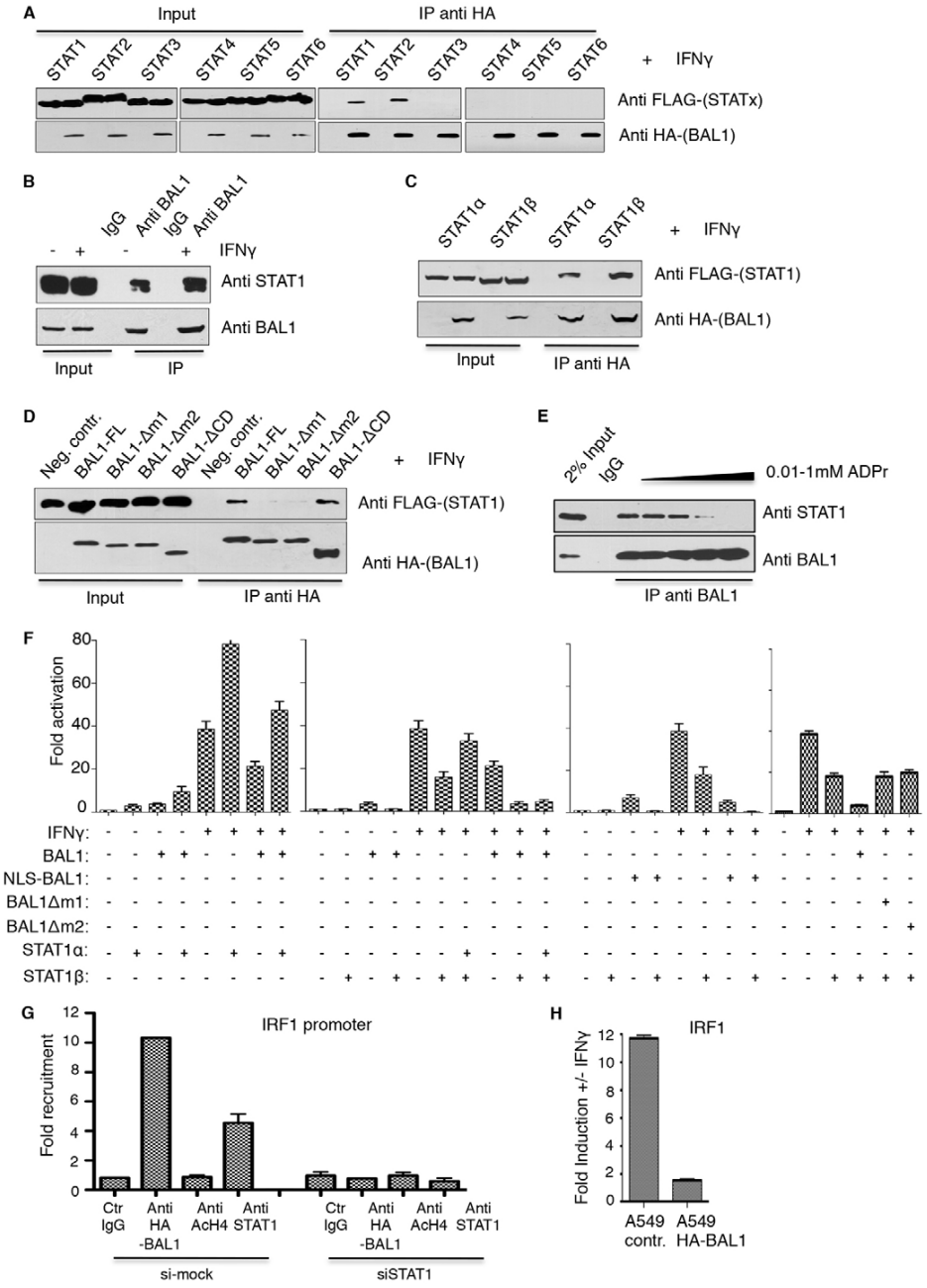

Fig. 4. See next page for legend.

mono-ADP-ribosylating ARTD family members (Hottiger et al., 2010; Wahlberg et al., 2012). We could not observe any effects on STAT1-dependent signaling and gene expression using Olaparib or Veliparib, two ARTD/PARP inhibitors highly specific to ARTD1 and ARTD2 (data not shown), also indicating that the enzymatic activity of ARTD1 and/or ARTD2 is not required for these BAL1/ARTD9-dependent processes.

Recent studies showed that overexpression of STAT1β, the antagonistic isoform of STAT1α increases the growth rate of cells and their resistance to drug-induced apoptosis and cell cycle arrest by repressing STAT1α target genes such as *p21* and *IRF1* in human B cells (Baran-Marszak et al., 2004). We therefore performed *IRF1*-promoter-driven luciferase reporter assays with BAL1, STAT1α and STAT1β, in the BAL1-negative DLBCL cell line U2932 (Fig. 4F). Indeed, coexpression of BAL1 wild-type or macrodomain deletion mutants together with STAT1α or STAT1β, respectively, along with an *IRF1*-promoter driven luciferase reporter in U2932 cells showed that overexpression of BAL1 wild-type together with STAT1β synergistically downregulated the *IRF1*-promoter driven luciferase reporter upon IFNγ stimulation, even in presence of STAT1α, whereas no BAL1-mediated repression was observed with BAL1-macrodomain deletion mutants, suggesting that the observed inhibitory effect is directly mediated through the macrodomain-dependent interaction of BAL1 with STAT1β (Fig. 4F). Next we tested whether STAT1 is required for the recruitment of BAL1 to its STAT1-dependent target promoters. Indeed chromatin immunoprecipitation analysis in A549 cells ectopically expressing HA-tagged BAL1 independent of STAT1 (supplementary material Fig. S3B,C) revealed that the recruitment of BAL1 to the *IRF1* promoter is strictly dependent on STAT1 (Fig. 4G,H).

Fig. 4. BAL1 forms an ADP-ribosylation-dependent complex with STAT1 and represses the IRF1 promoter together with STAT1β. (**A**) Co-immunoprecipitation of BAL1 and STATs overexpressed in HEK293 cells. HEK293 cells were co-transfected with expression vectors for HA-tagged BAL1 full length along with FLAG-tagged STAT1, STAT2, STAT3, STAT4, STAT5 or STAT6 and subsequently stimulated for 1 hour with 1000 U/ml IFNγ. HA–BAL1 and STAT1 complexes were then co-immunoprecipitated, separated on SDS-PAGE, blotted and subsequently probed with antibodies for HA (BAL1) and FLAG tag (STATs). (**B**) Interaction of endogenous BAL1 and STAT1 is partially dependent of IFNγ. SUDHL7 cells were stimulated for 1 hour with 1000 U/ml IFNγ and endogenous BAL1 and STAT1 complexes subsequently co-immunoprecipitated using an anti-BAL1 antibody. Complexes were then separated on SDS-PAGE, blotted and probed with antibodies against endogenous BAL1 and STAT1. (**C**) BAL1 interacts with both isoforms of STAT1. HEK293 cells were co-transfected with expression vectors for HA-tagged BAL1 full length along with FLAG-tagged STAT1α or STAT1β and subsequently stimulated for 1 hour with 1000 U/ml IFNγ. HA–BAL1 and STAT1 complexes were then co-immunoprecipitated, separated on SDS-PAGE, blotted and probed with antibodies for HA (BAL1) and FLAG tag (STATs). (**D**) Mapping of the interaction domains in BAL1. HEK293 cells were co-transfected with expression vectors for HA-tagged BAL1 full length, or deletion mutants [deletion of macro domain 1 and 2 (Δm1 and Δm2) or catalytic domain (ΔCD)] along with FLAG-tagged STAT1, and subsequently stimulated for 1 hour with 1000 U/ml IFNγ. HA–BAL1 and STAT1 complexes were then co-immunoprecipitated, separated on SDS-PAGE, blotted and probed with antibodies for HA (BAL1) and FLAG tag (STAT1). (**E**) Co-immunoprecipitation of endogenous BAL1 and STAT1 in the presence of ADP-ribose. SUDHL7 cells were stimulated for 1 hour with 1000 U/ml IFNγ and endogenous BAL1 and STAT1 complexes were subsequently co-immunoprecipitated in the presence of increasing concentrations of ADP-ribose (0.01–1 mM) using an anti-BAL1 antibody. BAL1-STAT1 complexes were then separated on SDS PAGE, blotted and subsequently probed with antibodies against endogenous BAL1 and STAT1. (**F**) BAL1 inhibits the *IRF1*-promoter-driven luciferase in U2932 cells. BAL1-negative U2932 cells were seeded in 12-well dishes at 0.4×10^6 cells/ml and co-transfected with an *IRF1*-promoter-driven luciferase reporter vector (500 ng DNA/ml) along with expression vectors for BAL1 wild type, 3x-NLS-BAL1 wild type or BAL1-macrodomain deletion mutants (BAL1Δm1 or BAL1Δm2), STAT1α (first and middle left) or STAT1β (middle left, middle right and right panels; 800 ngDNA/ml) and with the control reporter plasmid, pRL-hTK (100 ng/ml; TK-Renilla–luciferase control, Promega). The cells were then treated with 1000 U/ml IFNγ for 10 hours or left untreated. *IRF1*-promoter-Luciferase activities are normalized to the luciferase activities of the internal TK-Renilla–luciferase control and presented as means from 5 independent experiments performed in triplicate ± standard deviations. (**G**) ChIP analysis of IRF1 promoter for H4K16-acetylation, STAT1, HA–BAL1 and RNA-Pol-II recruitment in si-mock- and siSTAT1-RNA-treated A549 knockdown cells ectopically expressing HA-tagged BAL1 using anti-STAT1, anti-HA, anti RNA-Pol-II, anti-H4K16Ac and control (Ctr) antibodies. (**H**) *IRF1* gene expression analysis in A549 control cells and A549 cells ectopically expressing HA-tagged BAL1. *IRF1* mRNA levels were measured by qPCR and normalized against *GAPDH*.

BAL1 stimulates the phosphorylation and nuclear translocation of STAT1

We next investigated whether BAL1 could modulate the balance between transcriptionally active STAT1α and transcriptionally repressive STAT1β complexes in the nucleus through stimulation of STAT1α and STAT1β phosphorylation on tyrosine 701. Indeed, our phosphorylation analysis revealed that BAL1 stimulates the phosphorylation of both STAT1α and STAT1β on Y701 but not on serine 727 of the transcriptionally activating isoform STAT1α (Fig. 5A). IFNGR–JAK2-mediated phosphorylation of STAT1 on Y701 is required for STAT1 dimerization and its nuclear translocation (Darnell et al., 1994; Mowen and David, 2000). Phosphorylation on Y701 also enhances the nuclear shuttling by triggering the nuclear retention of the shuttling STAT1α and STAT1β, which are kept in the nucleus until tyrosine dephosphorylation occurs (Meyer et al., 2003). JAK2 is thought to phosphorylate STAT1 on Y701 in the cytoplasm, whereas JAK1 seems to be required for phosphorylation on Y701 in the nucleus, preventing nuclear export of STAT1 (Meyer et al., 2003; Mowen and David, 2000). However, phosphorylation on S727 in the transactivation domain of STAT1α can also occur independently of STAT1 tyrosine phosphorylation (Decker and Kovarik, 2000).

BAL1 might, therefore, mainly influence the shuttling kinetics of STAT1. Our subsequent subcellular fractionation analysis confirmed that BAL1 is indeed required for the constitutive exclusively nuclear localization of STAT1 in HR-DLBCL (Fig. 5B). Finally, our co-immunoprecipitation analysis revealed that BAL1 interacts with the IFNGR1 complex but not with the pY701-specific tyrosine phosphatase PTBN1 in HR-DLBCL (Fig. 5C), indicating that BAL1 may directly stimulate JAK2-mediated phosphorylation of STAT1 and thereby promote the nuclear accumulation of the antagonistically acting and transcriptionally repressive isoform STAT1β. Remarkably, a recent study provided the first evidence that the BAL1-related BAL2/ARTD8 promotes the survival of myeloma cells by inhibiting the kinase activity of c-Jun N-terminal kinase 1 (JNK1) (Barbarulo et al., 2012). Since phosphorylation of STAT1α on S727 is not affected by BAL1, our results indicate that BAL1 influences the nuclear activities of the transcriptionally repressive isoform STAT1β thereby tipping

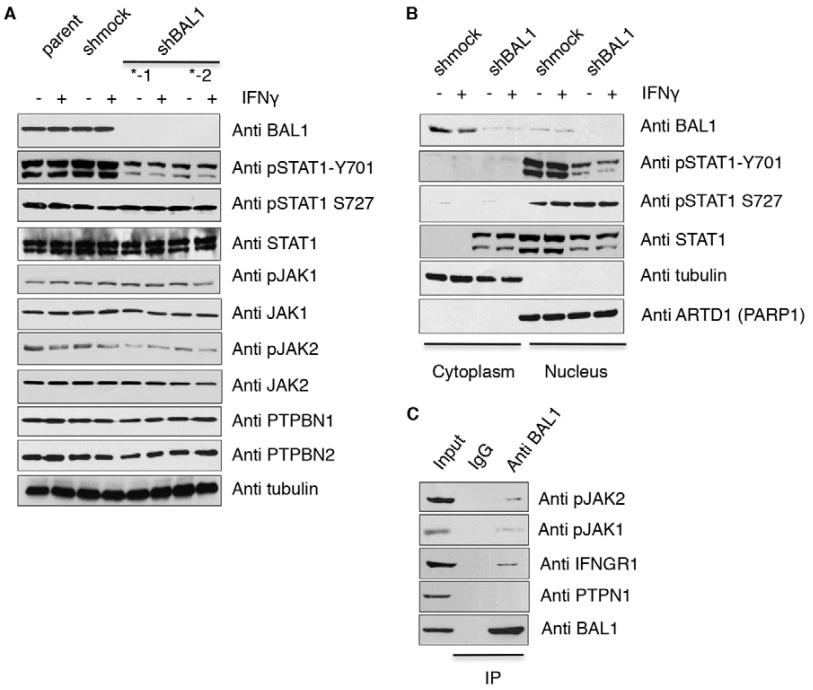

Fig. 5. BAL1 specifically stimulates the phosphorylation of STAT1α and STAT1β on Y701. (**A**) Immunoblot analysis of STAT1 signaling in SUDHL7-shmock and -BAL1-knockdown cells. Parental wild-type SUDHL7, shmock-RNA and *BAL1*-shRNA knockdown cells were untreated or treated with 1000 U/ml IFNγ for 8 hours and then whole-cell extracts were separated by SDS-PAGE, blotted and probed with antibodies for BAL1, STAT1, pSTAT1(Y701), pSTAT1(S727), JAK1, pJAK1, JAK2, pJAK2, PTPN1, PTPN2 and tubulin. (**B**) Subcellular fractionation analysis of STAT1 signaling in SUDHL7-shmock and BAL1-shRNA knockdown cells. The cells were untreated or treated with 1000 U/ml IFNγ for 8 hours and then nuclear and cytoplasmic cell extracts were separated by SDS-PAGE, blotted and probed with antibodies for BAL1, STAT1, pSTAT1(Y701), pSTAT1(S727), ARTD1(PARP1) and tubulin. (**C**) Co-immunoprecipitation analysis of endogenous BAL1-tyrosine-kinase/-phosphatase complexes in SUDHL7 cells. Endogenous BAL1/IFNGR complexes were co-immunoprecipitated using an anti-BAL1 antibody. Complexes were then separated by SDS-PAGE, blotted and probed with antibodies against endogenous BAL1, IFNγR1, pJAK1, pJAK2 and PTPN1.

the antagonistic balance between STAT1 dimers activating transcription and STAT1 dimers repressing transcription.

Discussion

In this study, we identified BAL1/ARTD9 acting as a novel IFNγ-specific oncogenic survival factor in high-risk DLBCL. We further showed that constitutive overexpression of BAL1 in DLBCL tightly associates with intrinsic IFNγ signaling and constitutive nuclear activity of STAT1. BAL1 counteracts the IFNγ-dependent anti-proliferative and pro-apoptotic IFNγ–STAT1–IRF1–p53 axis while concomitantly activating IFNγ/STAT1-dependent (i.e. IRF2-mediated) and IFNγ/STAT1-independent (i.e. BCL6/BCL2-mediated) anti-apoptotic-pro-survival pathways. As a consequence, overexpression of BAL1 in HR-DLBCL mediates cell proliferation chemo-resistance and survival in high-risk DLBCL. The observed inhibition of tumor suppressor IRF1 and simultaneous upregulation of the proto-oncogenes *IRF2*, *BCL2* and *BCL6* by BAL1 strongly correlates with the phenotype of high risk DLBCL *in vitro* and the clinical outcome of HR-DLBCL (Abramson and Shipp, 2005; Monti et al., 2005).

IRF1 mediates anti-proliferative and pro-apoptotic effects in cancer cells in a context-dependent and cell-type-specific manner (Choo et al., 2006). In the absence of antagonistic regulatory factors, increased expression and activation of IRF1 inhibits the expression of pro-survival members of the BCL2 family and simultaneously induces the expression and activation of pro-apoptotic and anti-proliferative gene products, including p53, p21 and CASP3 (Choo et al., 2006; Schwartz et al., 2011; Shi et al., 2011). IRF2, an antagonist of IRF1 is known to act as an oncogene product in various types of cancer and when overexpressed in cancer, IRF2 can abolish the tumor suppression function of IRF1 (Choo et al., 2006). Although loss of IRF1 alone is not associated with spontaneous tumor development in mice, it greatly increases tumor susceptibility in combination with loss of other tumor suppressor proteins such as p53 (Nozawa et al., 1999). On the other hand, it has been previously demonstrated that BCL6 can also suppress both, the basal and the induced expression levels of tumor suppressors p53 and the cyclin-dependent kinase inhibitor p21 (Phan and Dalla-Favera, 2004; Saito et al., 2009). Loss or mutation of p53 are observed in about 20% of patients with high risk DLBCL and have statistically significant negative impact on progression-free survival (Stefancikova et al., 2011). Loss or mutation of p53 is associated with a shorter survival after R-CHOP treatment (Stefancikova et al., 2011). Thus the observed BAL1-mediated downregulation of tumor-suppressors IRF1 and simultaneous upregulation of BCL6 represents another molecular mechanism inactivating the p53/IRF1 pro-apoptotic pathway in high-risk DLBCL-expressing wild-type p53.

Remarkably, our study provides the first evidence that BAL1 not only blocks IFNγ–STAT1–IRF1-mediated apoptosis and inhibition of growth, but also reverses the chemo-resistance in high-risk DLBCLs. Our results also strongly indicate that STAT1 acts as an oncogene in high-risk HR-DLBCL with an active host inflammatory response. This activity is at least partially mediated by BAL1. BAL1 facilitates the oncogenic functions of STAT1 by counteracting the pro-apoptotic IFNγ–STAT1–IRF1 axis. Moreover, together with STAT1β, BAL1 may negatively regulate a tumor suppressor network, thereby inducing a switch in STAT1 from a tumor suppressor to an oncogene. This also explains why constitutive IFNγ–STAT1 signaling does not lead to apoptosis but rather to survival and chemo-resistance in HR-DLBCL (Fig. 6). Recent studies showed that aberrant nuclear localization and activity of STAT1 leads to radio- and chemo-resistance in solid cancers (Khodarev et al., 2004; Khodarev et al., 2009; Khodarev et al., 2012; Stronach et al., 2011; Weichselbaum et al., 2008).

Strikingly, our studies also demonstrate that BAL1 interacts with the IFNGR complex and enhances tyrosine phosphorylation

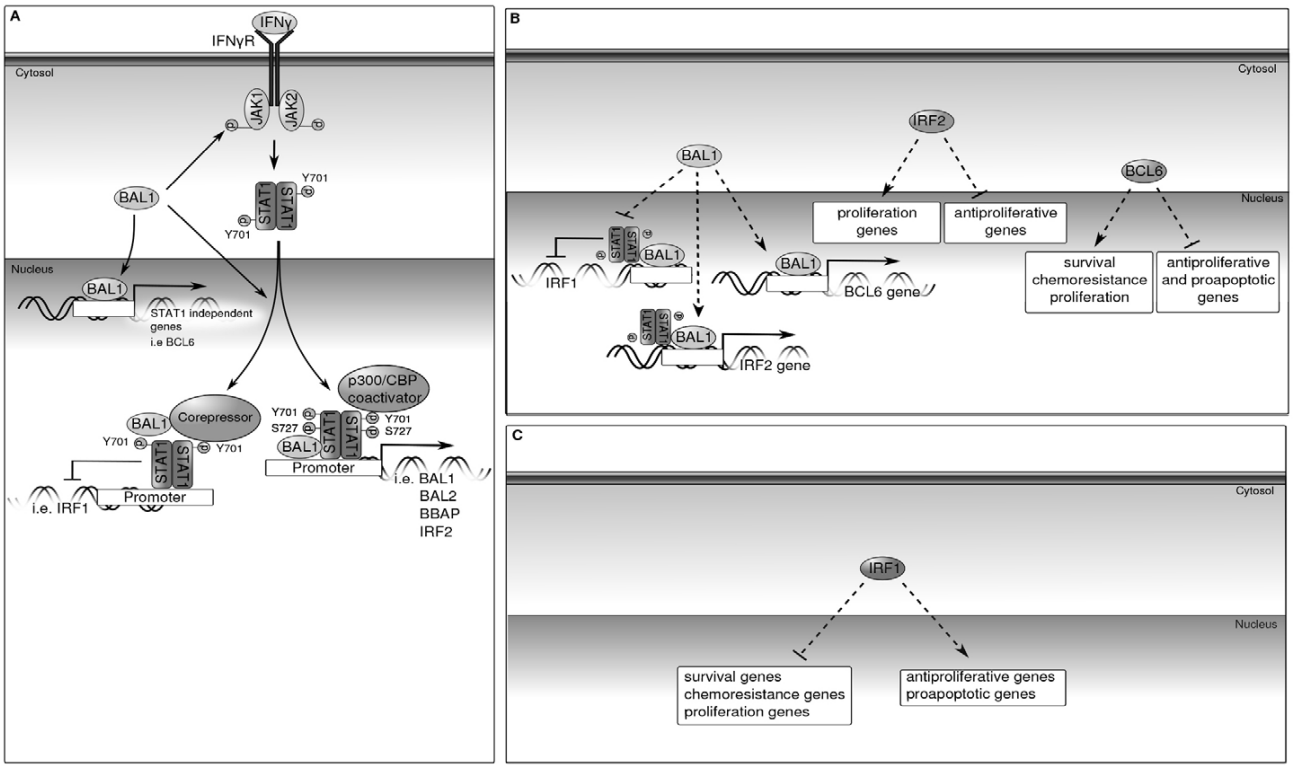

Fig. 6. Model for the BAL1-mediated oncogenic switch in STAT1 in HR-DLBCL. (**A**) Constitutively active IFNγR-JAK1/2-STAT1 signaling in HR-DLBCL causes overexpression of BAL1, which in turn further stimulates the phosphorylation of STAT1 on Y701 and subsequently the enhanced expression of STAT1α-dependent proto-oncogenes (i.e. *BBAP*/*BAL1*, *BAL2*, *IRF2*). Conversely, BAL1 together with STAT1β represses the transcriptional activation of tumor suppressor IRF1. In addition, overexpression of BAL1 also enhances the expression of STAT1-independent proto-oncogenes (i.e. *BCL6*). (**B**) Overexpression of BAL1 prevents intrinsic and extrinsic IFNγ-STAT1-IRF1/p53-mediated cell death pathways while simultaneously enhancing the STAT1-dependent IRF2-mediated proliferation and STAT1-independent BCL6–BCL2-mediated survival pathways. As a consequence BAL1 induces an switch in STAT1 from a tumor suppressor to an oncogene and mediates proliferation, survival and chemo-resistance in HR-DLBCL. (**C**) Inactivation of BAL1 in HR-DLBCL associated with constitutively active STAT1 signaling reactivates the anti-proliferative and pro-apoptotic IFNγ-STAT1-IRF1/p53 axes and reverses chemo-resistance in HR-DLBCL.

of both STAT1 isoforms, STAT1α and STAT1β, on Y701, and their subsequent nuclear translocation, thereby promoting the nuclear accumulation of the antagonistically acting and transcriptionally repressive isoform STAT1β. Thus, BAL1 influences the nuclear activities of the transcriptionally repressive isoform STAT1β, tipping the antagonistic balance between STAT1 dimers activating transcription and STAT1 dimers repressing transcription. However, the exact molecular mechanism(s) underlying the isoform- and promoter-specific regulation of STAT1 by BAL1 remain to be elucidated in future studies. Unfortunately, the specific antagonistic nuclear activities of STAT1α and STAT1β, and their distinct phosphorylated and unphosphorylated forms in particular, are in general not very well investigated yet.

In contrast, *BCL6* is not likely to be a direct target gene of STAT1, so the observed BAL1-mediated stimulation of BCL6 expression might be caused by another unknown mechanism. BCL6 overexpression is mediated through multiple mechanisms in DLBCL: translocation, hypermutation of its promoter, or inactivation of FBXO11, which results in increased levels and stability of BCL6 (Duan et al., 2012). Transcriptional upregulation of *BCL6* by BAL1, therefore, represents an additional molecular mechanism for overexpression of BCL6 in DLBCL. Moreover, the observed concomitant overexpression of BCL2 and BCL6 in SUDHL-7 cells also indicates that BAL1 could block BCL6-mediated repression of the *BCL2* gene, which is frequently disrupted in DLBCL (Saito et al., 2007; Saito et al., 2009). However, the exact STAT1-independent molecular mechanisms involved in BAL1 stimulation of the transcriptional activation of *BCL6* and *BCL2* have to be thoroughly investigated in future.

Finally our study provides the first evidence for an IFNγ-dependent STAT1–BAL1–BCL6-mediated anti-apoptotic-, pro-survival-regulatory circuit in HR-DLBCL and explains why STAT1 could function as an oncogene in a subset of HR-DLBCL. In addition, our observations could also provide a molecular mechanism for the risk-related activity of BAL1 in HR-DLBCL subsets without constitutive active STAT1 signaling. BAL1 could be directly involved in editing or inhibiting the IFNγ-dependent host immune response against HR-DLBCL through the termination of IFNγ-mediated gene expression and inhibition of the extrinsic IFNγ-induced

anti-proliferative and pro-apoptotic STAT1–IRF1–p53 axes. The observed macrodomain and ADP-ribosylation-mediated interaction between BAL1 and STAT1 as well as the BAL1-mediated upregulation of BAL2/ARTD8 also indicates a regulatory cross talk between BAL1 and other active members of the ARTD family such as BAL2/ARTD8 or ARTD10 in these processes. BAL2/ARTD8 is a macrodomain-containing ARTD family member and an active mono-ADP-ribosyltransferase mediating survival in c-Myc-driven Burkitt lymphoma-like tumor cells *in vivo* (Cho et al., 2009a). BAL2/ARTD8 has been suggested to function as a STAT6-specific co-regulator of IL-4-mediated gene expression and has been suggested to be involved in mediating IL-4-induced proliferation and protection of B-cells against apoptosis following irradiation or growth factor withdrawal (Cho et al., 2009b; Goenka and Boothby, 2006; Goenka et al., 2007).

Together, our studies further strengthen the hypothesis that BAL1 may serve as a novel potential drug target for treatment of high-risk chemo-resistant diffuse large B-cell lymphoma. The combination of classic therapeutic drugs with novel drugs targeting STAT1 or the macrodomains of BAL1/ARTD9 might be a strategy to increase the sensitivity of HR-DLBCL towards classic therapy, and thus pave the way to develop novel therapeutic strategies for the remainder of DLBCL patients suffering from aggressive chemo-resistant high-risk host response variants of DLBCL.

Material and Methods

Cell culture, transfections, luciferase reporter assays and generation of stable cell lines

The human lung carcinoma cell line A549, 293HEK and DLBCL cell lines were cultured as described previously (Hassa et al., 2005; Saito et al., 2009). The DLBCL cell lines SUDHL2, SUDHL4, SUDHL6, SUDHL7, U2932, OCI-Ly1, OCI-Ly3 and OCI-Ly10 were provided by Dr Riccardo Dalla-Favera (Columbia University, NY, USA), Dr Jose Martinez-Climent (Spanish National Cancer Research Centre, Madrid, Spain) and Dr Louis Staudt (National Institutes of Health, Bethesda, MD, USA). 293HEK were purchased from ATCC. Transfections of cells with plasmid DNA (for reporter assays and generation of stable cell lines) were performed with Fugene HD, Extreme gene 9 and HP transfection reagents (Roche Applied Science) according to the manufacturer's protocols. Stable cell lines were generated using a Piggyback transposon and a BIC-miR155-precurser-RNA based shRNA-expressing system. Piggyback-transposed cells were selected with puromycin (500 ng/ml). Transfections of siRNA oligos (40 pmol siRNA/transfection) were performed with Lipofectamine RNAimax reagent (Invitrogen) or Extreme gene siRNA (Roche Applied Science) according to manufacturers' protocols. Luciferase reporter assays were performed as previously described (Hassa et al., 2005) and according to manufacturer's protocols (Promega) using the Dual Luciferase assay kit (Promega) and a TECAN infinite M200 luminometer (Tecan Systems).

Plasmids

Human BAL1/ARTD9 cDNA was amplified by PCR from a B-cell lymphoma cDNA library and cloned into the corresponding expression vectors (pcDNA-HA-, pPiggyBac-EF1aprom-HA-BAL1) using *Nhe*I–*Not*I or *Not*I, respectively. BAL1-domain deletion and GST-fusion constructs were generated by PCR and cloned into the *Nhe*I–*Not*I or *Eco*RI–*Not*I sites of pcDNA-HA- and pETM-GST-MCS1, respectively. All constructs and full-length cDNA sequences were verified by sequencing. All empty basic Piggyback transposon vectors and expression vectors for the latest version of Piggyback transposases were either purchased from System Biosciences (SBI) Inc. (Mountain View, CA, USA) or provided by Dr Allan Bradley (Wellcome Trust Sanger Institute, UK). The BIC-mir155 vector system (Chung et al., 2006) was provided by Dr David L. Turner (Molecular and Behavioral Neuroscience Institute, University of Michigan, Ann Arbor, USA). The siRNA and shRNA/DNA oligos were purchased from Qiagen. The corresponding siRNA and shRNA sequences are listed in supplementary material Table S1. FLAG–STATs were purchased from Addgene. *hIRF1*-prom-luciferase reporter vectors were a gift from Dr R. Pine (Public Health Research Institute, Newark, NJ, USA).

Reagents

Human recombinant IFNγ was purchased from PeproTech, doxorubicin and etoposide were purchased from Sigma. Tosyl-//activated Dynabeads were purchased from Invitrogen. ADP ribose was purchased from Sigma. High-performance glutathione–Sepharose and Ni-Sepharose were purchased from Amersham Biosciences.

Gene expression analysis

Real-time qPCR analysis was performed essentially as described previously (Guetg et al., 2012). Total RNA was isolated using Trizol (Invitrogen) or Tri-Reagent (MRC Inc.) according to manufacturers' protocols. RNA was subsequently reverse-transcribed using the 'High-Capacity cDNA Reverse Transcription kit' (Applied Biosystems) according to manufacturer's protocols. Real-time qPCR was performed using the Rotor-Gene 3000 (Corbett Life Science, now Qiagen) and SYBR Green kit (Bioline) according manufacturers' protocols using the primers listed in supplementary material Table S2. Mean values ± s.e.m. were calculated and plotted as graphs with GraphPad Prism (GraphPad Software).

Chromatin immunoprecipitation

Cells were collected and crosslinked with 1% formaldehyde as described previously (Covic et al., 2005). Chromatin fragmentation was achieved with the Bioruptor (Diagenode). Antibodies were incubated with crosslinked chromatin overnight at 4℃ and collected with Protein-A agarose/salmon sperm DNA (Millipore) for 3 hours. After reversal of the crosslinking and digestion with proteinase K, DNA was extracted and measured by real-time PCR using SYBR Green and the Rotor-Gene 3000 (Corbett Life Science/Qiagen).

Expression, purification of recombinant proteins

Recombinant HIS–GST–BAL1 fusion proteins were expressed in *E. coli* strains BL21 DE3-Rosetta-II, BL21 DE3-Rosetta-II-Tuner and BL21-ArcticExpress-Rosetta-II as described previously (Timinszky et al., 2009). All purified proteins were analyzed using Coomassie Blue staining and confirmed by western blot analysis using the corresponding antibodies.

Interaction assays, immunoblot analysis and immunofluorescence microscopy

Membrane, cytoplasmic, nuclear and whole-cell extracts were prepared as described previously (Cunningham et al., 2003; Dignam et al., 1983; Hassa et al., 2005; Okada et al., 2008; Sen et al., 2011) with minor modifications: membrane extraction buffer contain 50 mM HEPES (pH 7.9) 150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 1 mM EDTA, 1 mM EGTA and cOmplete, EDTA-free Protease Inhibitor Cocktail (Roche). For immunoprecipitation, membrane and cytoplasmic extract fractions were re-mixed. Co-immunoprecipitation and GST pull-down assays were performed as described previously (Hassa et al., 2005; Owen et al., 2005; Timinszky et al., 2009), except that the antibodies used for immunoprecipitation were covalently coupled to tosyl-activated Dynabeads (Invitrogen) according to the manufacturer's protocols. Immunoprecipitation buffers contained 80 mM Tris (pH 7.05), 125 mM NaCl, 25 mM potassium acetate, 1.5 mM $MgCl_2$, 5% glycerol, 0.5% NP-40, 0.5 mM DTT, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 0.1 mM Na_3VO_4, and cOmplete, EDTA-free Protease Inhibitor Cocktail (Roche). GST pull-down buffers contained 80 mM Tris (pH 7.05), 1.5 mM $MgCl_2$, 150 mM NaCl, 50 mM potassium acetate, 0.5% NP-40, 0.5 mM DTT, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 5% glycerol and cOmplete, EDTA-free Protease Inhibitor Cocktail (Roche). Immunoblot procedures and immunofluorescence microscopy were performed as described previously (Guetg et al., 2012; Hassa et al., 2005) using the following primary antibodies: monoclonal mouse anti-HA and mouse anti-FLAG antibodies (both Sigma), polyclonal rabbit anti-BAL1 antibodies (C-terminal; Millipore), monoclonal rabbit anti-STAT1, anti-pSTAT1(Y701), anti-pSTAT1(S727), anti-STAT2, anti-pSTAT2(Y690), anti-IRF2, anti-BLIMP1, anti-Casp3, anti-BCL2, anti-BAD, anti-BAD-S112, anti-PIM1, anti-PIM2, anti-PTBN1, anti-JAK1, anti-pJAK1, anti-JAK2, anti-pJAK2, anti-IFNGR1, anti-PTBN2 and anti-BCL-XL antibodies (RabMab, Epitomics), monoclonal rabbit anti-IRF1 (RabMab, Cell Signaling Technology), monoclonal mouse anti-tubulin, polyclonal rabbit anti-p53 and anti-BCL6 antibodies (Santa Cruz Biotechnology). The polyclonal rabbit anti-BAL2/ARTD8 antibody was a generous gift from Avraham Raz (Karmanos Cancer Institute, School of Medicine, Wayne State University, Detroit, Michigan 48201, USA) (Yanagawa et al., 2007). Immunofluorescently stained cells were analyzed by fluorescence microscopy on a Leica DMI6000B automated inverted research microscope system (Leica Microsystem). Composite images were generated using Adobe Photoshop software.

Flow cytometry analysis, survival and proliferation assays

FACS analysis was performed with the following PE-conjugated antibodies (Biolegend) according to manufacturer's protocols: PE anti-human CD119 (IFNγR α-chain) PE anti-human CD40, PE Syrian hamster IgG isotype control and PE mouse IgG2 isotype control antibodies. PE-positive and PE-negative cells were

sorted using a BD FACSVantage cell sorter flow cytometer (BD Biosciences). Cell viability and proliferation were assessed by Trypan Blue exclusion and by the WST1 assays (Roche Diagnostics) according to manufacturers' protocols. For the cell viability and proliferation assays cells were seeded at 0.2×10^6 cells/ml in six-well dishes 8 hours prior to initiation of treatment and then incubated in the presence of PBS, DMSO (mock-treated), etoposide (20 μM) or doxorubicin (2 μM) for 24 hours. After 24 hours the cultures were washed with medium prior to reseeding in fresh medium for continued culture (24, 48, 72 and 96 hours). Relative cell viability/proliferation and cell numbers are presented as means from three independent experiments performed in triplicate ± standard deviations.

Analysis of tissue samples

In total, 250 formalin-fixed and paraffin-embedded tissue samples were analyzed, which consisted of 12 tonsils with reactive lymphoid hyperplasia and 238 diffuse large B-cell lymphomas (DLBCL). Histopathological diagnosis was rendered according to the World Health Organization classification. Two cores of 0.1 cm diameter were punched out of each sample of the respective donor blocks and transferred to Tissue-Micro-Array recipient blocks, from which 3 μm thick sections were cut and mounted on Superfrost slides (Menzel Glaser). The primary antibodies used were anti-pSTAT1(Y701) (rabbit polyclonal, Cell Signaling Technology, dilution 1:25 in Ventana Buffers), anti-pSTAT1(S727) (rabbit monoclonal, Epitomics Inc., dilution 1:400 in Ventana Buffers) and anti-IRF2 (rabbit monoclonal, Epitomics Inc., dilution 1:50 in Ventana Buffers). Staining was performed with the Ventana Discovery Ultra automated staining system (Ventana Medical Systems Inc.), operated according to the manufacturer's instructions and using solely Ventana reagents during the procedure. Detection was performed with the ChromoMap DAB detection kit using Ultra Map Anti Rb HRP. Negative controls were performed by omission of the primary antibodies. Slides were counterstained with Hematoxylin, dehydrated and mounted. Staining intensity was assessed with a light microscope, allocating a semi-quantitative score to each core. For all three markers, the approximate percentage of stained nuclei was recorded. The scoring was undertaken as follows: $<10\%$ was scored as 1, 10–50% as 2, 50–80% as 3 and $>80\%$ as 4; negative staining was scored as 0. Scoring was omitted for cores not containing appropriate tissue or in cores with artifacts resulting from crushing (see also supplementary material Tables S3, S4).

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Author contributions

R.C., H.C.W., S.B.B. and P.O.H. designed the experiments and analyzed results. R.C., H.C.W., S.B.B. and P.O.H. performed the research. M.T., E.H. and M.B. performed the evaluation of clinical samples. R.C. and P.O.H. wrote the paper. P.O.H. designed and supervised the research study. H.C.W. and S.B.B. contributed equally to the paper. All the authors read and corrected the manuscript.

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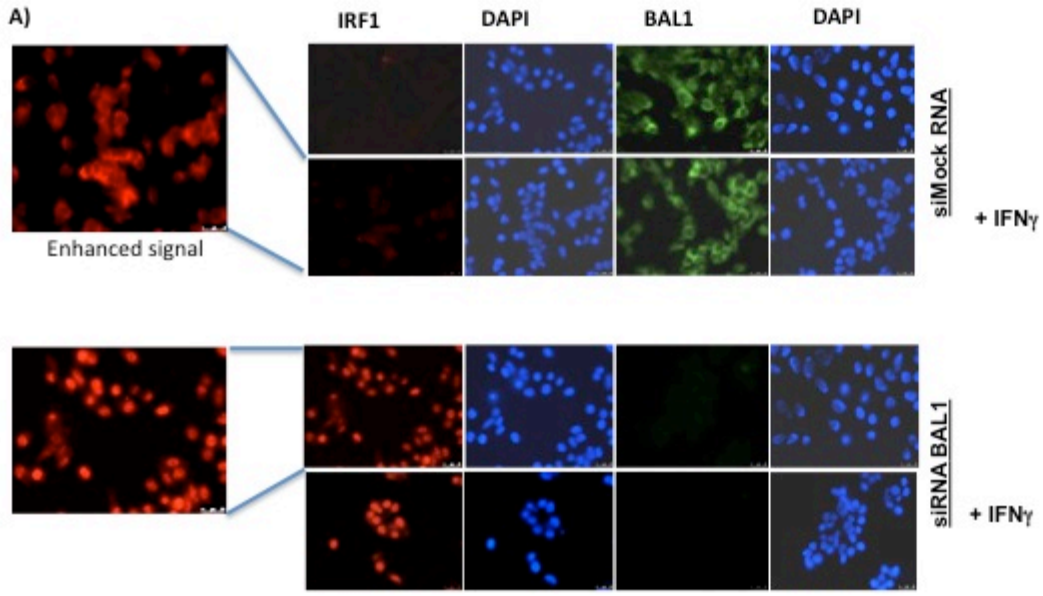
4.2 Unpublished results

4.2.1 BAL1/ARTD9 inhibits the nuclear translocation of tumor suppressor IRF1

Since BAL1/ARTD9 is a nucleo-cytoplasmic shuttling protein, we next investigated whether BAL1/ARTD9 could also regulate the sub-cellular localization of IRF1. Surprisingly, immuno-fluorescence analysis for BAL1/ARTD9 and IRF1 in SUDHL7-BAL1/ARTD9-shRNA knockdown cells revealed that BAL1/ARTD9 could also repress IRF1 at the level of nuclear translocation (Figure 8A). The remaining poorly expressed IRF1 in parental and si-mockRNA transfected SUDHL7 cells does not translocate to the nucleus and accumulates in the cytoplasm upon IFNγ stimulation (Figure 8A). Conversely IRF1 localizes to the nucleus in absence of BAL1/ARTD9 in SUDHL7-BAL1/ARTD9-siRNA knockdown cells upon IFNγ stimulation (Figure 8A). The major cytoplasmic localization of BAL1/ARTD9 in the BBAP/DTX3L expressing SUDHL-7 cell line is in agreement with previous studies demonstrating that both BAL1/ARTD9 in the BBAP/DTX3L proteins are mainly localized in the cytoplasm and less prominent, but significantly expressed in the nucleus of primary HR-DLBCL (25) It has been therefore suggested that complex formation between BBAP/DTX3L and BAL1/ARTD9 in the nucleus could facilitate the nuclear export of the BAL1/ARTD9 by BBAP/DTX3L (25) and thus tightly regulate the nuclear function of BAL1/ARTD9 in HR-DLBCL (Fig.9A). However the molecular mechanism(s) underlying the observed effects remain(s) to be elucidated. Surprisingly, control experiments showed that BAL1/ARTD9 regulates the nuclear translocation or nuclear export of IRF1 in an IFNγ-dependent and opposing manner (Fig. 9B). Constitutive ectopic expression of BAL1/ARTD9 in the macrophage-like lung alveolar (type-II) epithelial cell line A549, which is also used as model cell line for IFN-signaling, surprisingly revealed that BAL1/ARTD9 induces the expression

and nuclear translocation of IRF1 in absence of IFNγ and BBAP/DTX3L. Conversely, IRF1 was mainly detectable in the cytoplasm and on the periplasmatic membrane upon IFNγ stimulation, strongly indicating that another factor such as BBAP/DTX3L is required for the observed BAL1/ARTD9 dependent inhibition of nuclear translocation of IRF1 in DLBCL (Fig. 9B). However the molecular mechanism(s) underlying the observed effects remain(s) to be elucidated.



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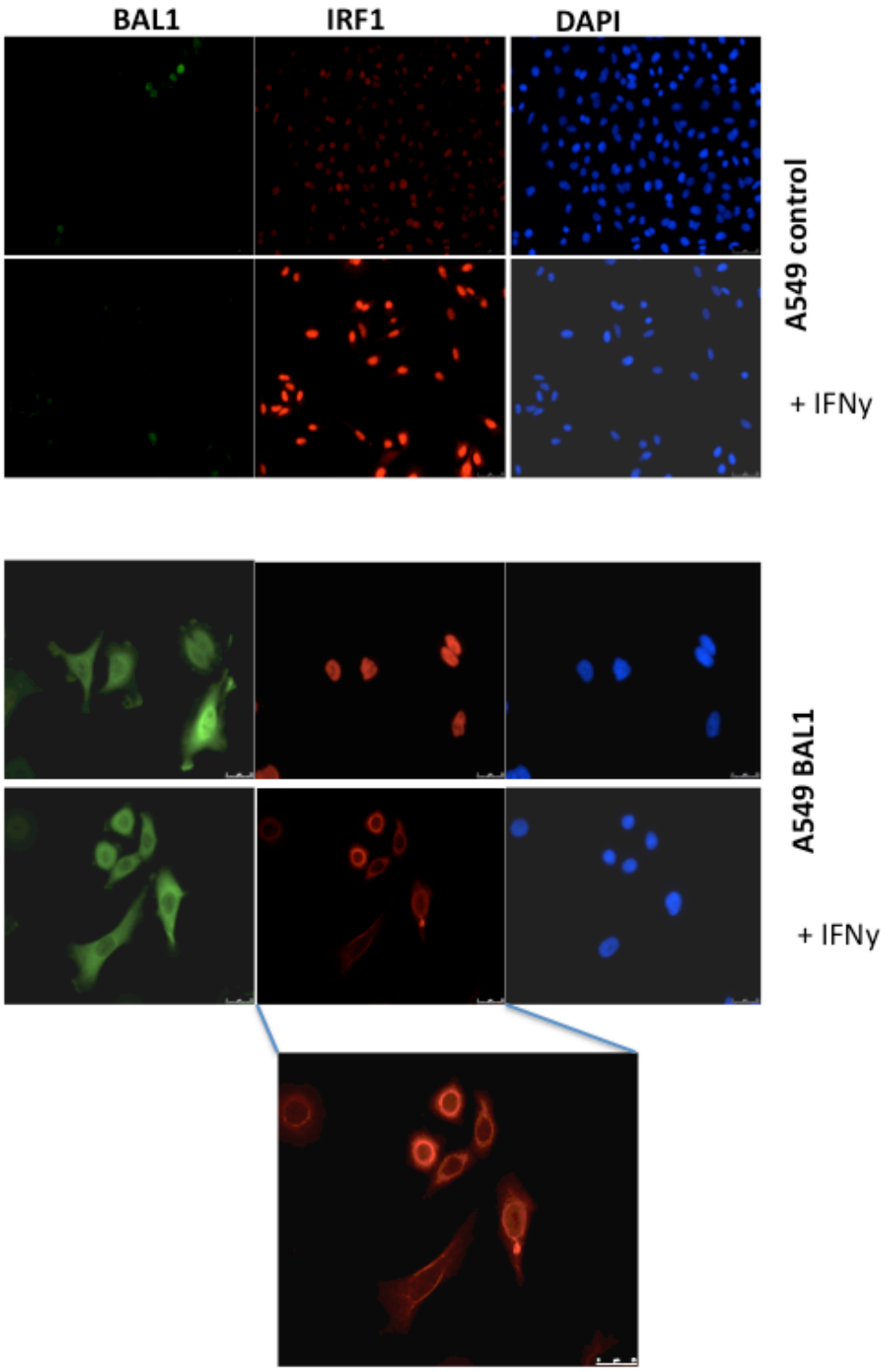


Figure 9. BAL1/ARTD9 inhibits the nuclear translocation of tumor suppressor IRF1. A) Immunofluorescence microscopy and sub-cellular localization of endogenous IRF1 and BAL1/ARTD9 in transient siRNA-BAL1 and simock RNA SUDHL7 knockdown cells, in presence or absence of IFNγ. **(B)** Immunofluorescence

microscopy and sub-cellular localization of endogenous IRF1 in BAL1 ectopically over-expressing and control A549 cell lines (co-staining using mono anti-BAL1 and poly anti-IRF1 antibodies).

4.2.2 Lack of BAL1/ARTD9 correlates with low IRF2 nuclear expression levels

The antitumor activity of IFNγ is mainly mediated through IRF1 and blocked by IRF2 (107, 108). IRF1 and IRF2 play antagonist roles in cancer, which is best described in breast cancers (180-182). In many breast cancers IRF1 is mainly inactive and localized in the cytoplasm, while IRF2 is highly expressed in the nucleus and enhances the tumor growth (107, 108, 180-182). We therefore investigated the potential antagonistic role of IRF1 and IRF2 in DLBCLs and could observe the same correlation as observed in breast cancer (**Fig. 10**). Lack of BAL1/ARTD9 correlates with low IRF2 nuclear expression levels, although the nuclear translocation of IRF2 is not regulated by BAL1/ARTD9 in DLBCL (**Fig.10**).

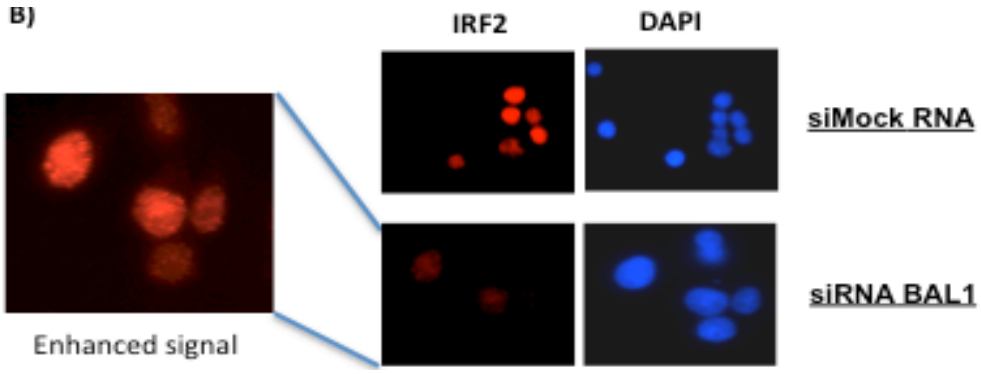


Fig.10 Lack of BAL1/ARTD9 correlates with low IRF2 nuclear expression levels. **A**) Immunofluorescence microscopy and sub-cellular localization of endogenous IRF2 in transient si-BAL1/ARTD9-RNA and simock-RNA SUDHL7 knockdown cells, in presence or absence of IFNγ.

4.2.3 Auto-Regulation of the bidirectional BAL1/BBAP promoter by positive and negative feedback loop mechanisms in DLBCL

BBAP/DTX3L was originally identified as a binding partner of BAL1/ARTD9 (163). BBAP/DTX3L is also over-expressed in subtypes of high risk chemotherapy-resistant "host response" HR-DLBCL (25, 163). In order to investigate whether BAL1/ARTD9 and BBAP/DTX3L are able to regulate each other also on the level of gene expression through their bidirectional BAL1/BBAP promoter we analyzed their expression levels in shBAL1-RNA and shBBAP-RNA SUDHL7 cell lines as well as in BAL1/ARTD9 and BBAP ectopically expressing GCB- and ABC-DLBCL cell lines. Surprisingly these results revealed that BAL1/ARTD9 enhances both basal and IFNγ-induced expression of BBAP/DTX3L and BAL2/ARTD8 in the HR-DLBCL cell line SUDHL7, which endogenously expresses BAL1/ARTD9 and BBAP/DTX3L. Conversely ectopic expression of BAL1/ARTD9 in BAL1/ARTD9-negative GCB- and ABC-DLBCL cell lines stimulates basal expression of BBAP/DTX3L and BAL2/ARTD8 while repressing the IFNγ-induced expression of BBAP/DTX3L and BAL2/ARTD8 in these cell lines. These results strongly indicate that BAL1/ARTD9 and BBAP/DTX3L are able to regulate each other in a cell type specific manner through positive and/or negative feedback loop mechanisms of auto-regulation (**Fig. 11**).

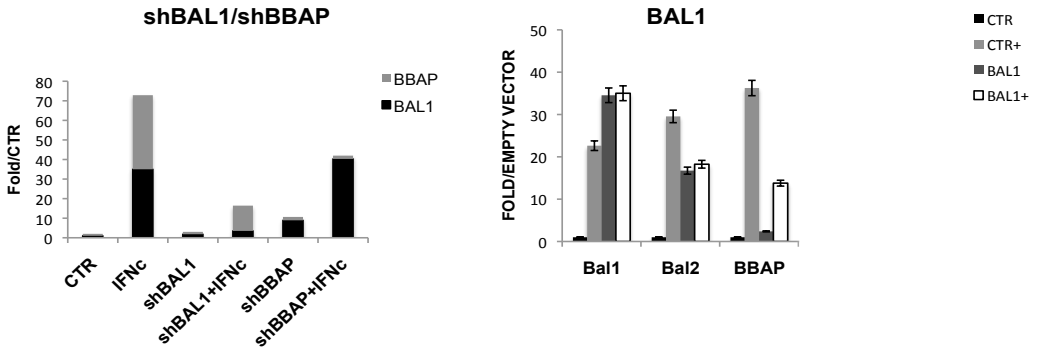
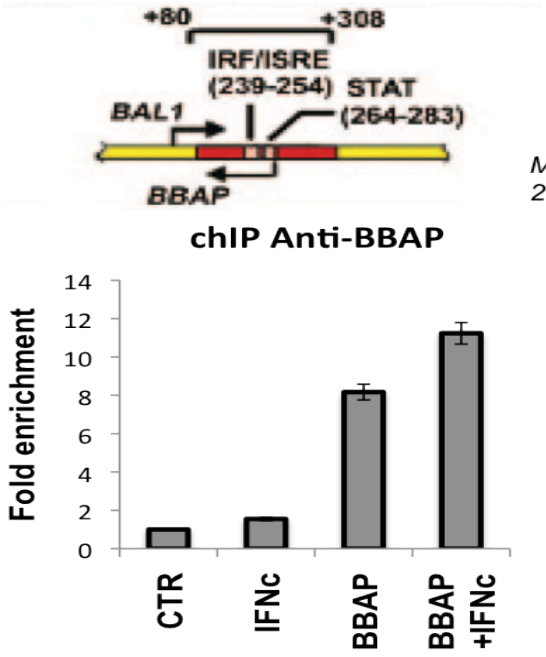


Figure 11. mRNA expression levels analysis of BAL1/ARTD9 and BBAP/DTX3L by q-PCR.
mRNA was isolated from mock,shBAL1-RNA and shBBAP-RNA expressing cell lines and compared with control, BAL1/ARTD9 or BBAP/DTX3L over-expressing cell lines. mRNA levels were measured by q-PCR and normalized against GAPDH and presented here as fold on the control cell lines.

To further assess whether this regulation is occurring directly on the chromatin levels, we performed ChIP assays in order to test whether both BAL1/ARTD9 and BBAP/DTX3L are recruited to their own bidirectional promoter. Indeed, these experiments revealed that also BBAP/DTX3L binds its own bidirectional promoter leading to the conclusion that BAL1/ARTD9 strictly associates with BBAP/DTX3L expression levels and auto-regulates their own expression in cell type and condition dependent manner (**Fig.12**).



Mol Cell Biol. 2006 July; 26(14): 5348–5359.

Figure 12. BBAP/DTX3L is also recruited to its own bidirectional promoter and associates with BBAP/DTX3L expression levels.

4.2.4 Does BAL1/ARTD9 stimulate growth, migration and chemo- and radio-resistance in prostate cancer?

Recent gene expression studies have revealed that IFN signaling may play an important role in human prostate cancer (118, 119). For instance, 29% of clinical human prostate cancers analyzed, constitutively expressed STAT1 and interferon stimulated genes (ISGs) in vivo (118). STAT1 has been therefore suggested as a potential target for radio-sensitization of aggressive tumors that constitutively over-express IFN/STAT1-dependent pathways. It has been shown that multiple small doses of ionizing radiation can activate an IFN (α, β, and γ)-related, STAT1-dependent DNA damage gene expression signature (including STAT1, G1P2, G1P3, IFITM1, IFIT1, IRF9, MX1, HLA-C, OAS1 and OAS3) in prostate cancer (117-121). Moreover, in vitro selection against IFNα or IFNγ and constitutive expression of

STAT1 leads to an IFN- and radio-resistant phenotype in prostate tumor cells (117, 118). In addition, the IFN/STAT1 signaling pathway is also up-regulated in chemo-resistant prostate cancer cells (118, 119). Remarkably, STAT1-dependent chemo-resistance was also associated with increased resistance to ionizing radiation and accompanied by the up-regulation of ISGs that overlapped, in part, with the IFN/DNA damage gene expression signature (117, 118). However, the exact molecular mechanisms how STAT1 signaling pathways mediate chemo- and radio-resistance in prostate cancer remain to be elucidated. We therefore investigated whether BAL1/ARTD9 could play a similar role in prostate cancer, thus being at least in part responsible for the STAT1 mediated chemo- and radio-resistance observed in prostate cancer. In an initial screening done in our lab we observed that BAL1/ARTD9, BAL2/ARTD8 and BBAP/DTX3L, are also constitutively over-expressed in different metastatic prostate carcinoma cell lines, including the semi-chemoresistant, p53 negative and androgen-refractory metastatic prostate cancer cell lines PC3 and DU145 (183-187) (personal communications, S.B. Bachmann and P.O. Hassa). Moreover, both PC3 and DU145 cell lines are also associated with constitutive STAT1 signaling and nuclear localization of pSTAT1-S727 (**Fig. 13**).

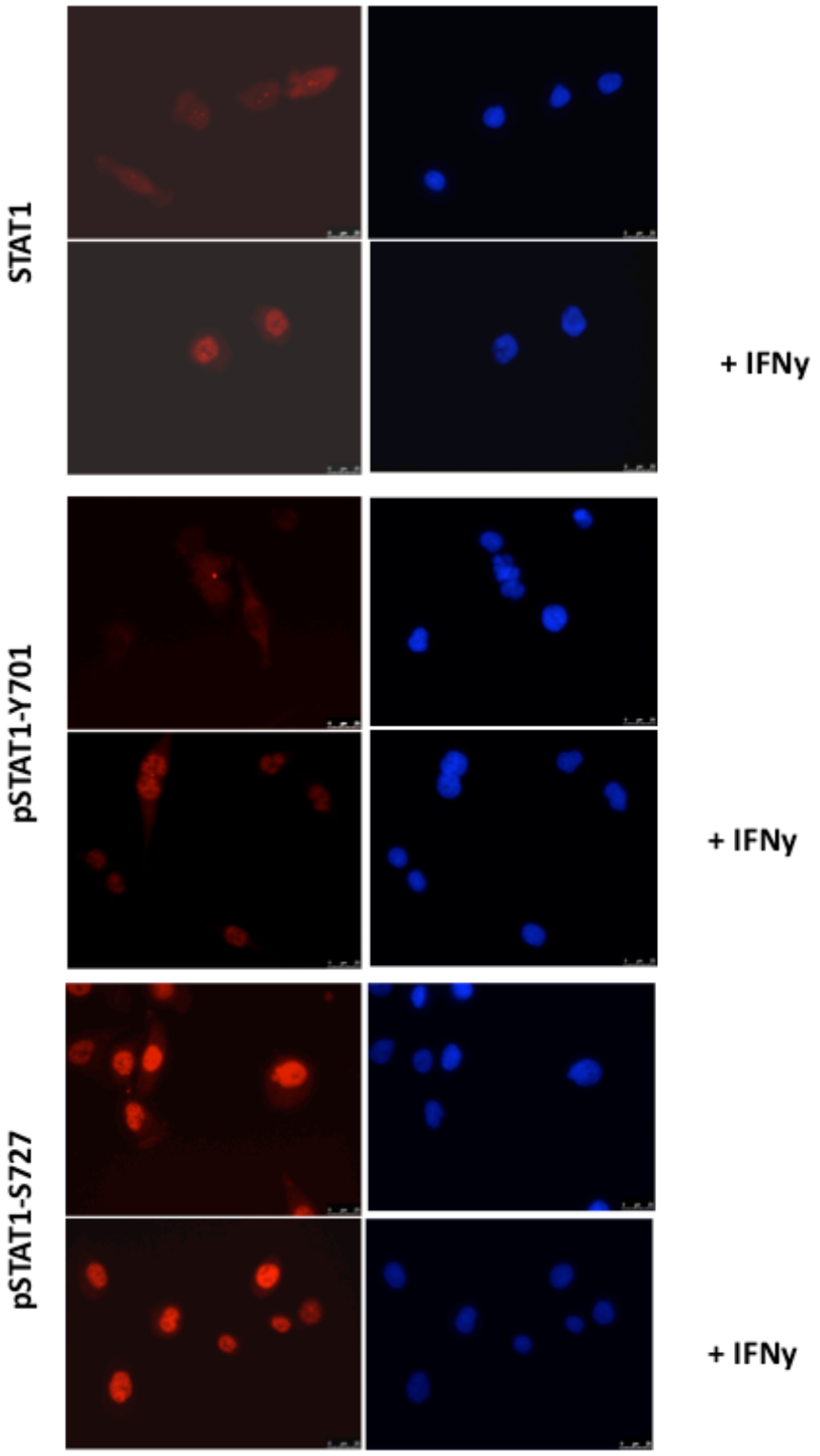


Fig.13 PC3 cells are associated with constitutive STAT1 signaling.
Immunofluorescence microscopy and sub-cellular localization of endogenous STAT1, pSTAT1-Y701 and pSTAT1-S727 in presence or absence of IFNγ.

Our preliminary and ongoing experiments already revealed that BAL1/ARTD9 could indeed play a similar survival related function in androgen-refractory metastatic prostate cancer as previously observed in chemo-resistant HR-DLBCL cells. Expression analysis of IRF1 in PC3-siRNA-BAL1/ARDT9 knockdown cells showed that IRF1 is up-regulated in absence of BAL1/ARDT9 when compared with siRNA mock cells (Figure 13). We next compared the phosphorylation pattern of STAT1 in PC3-siRNA-BAL1/ARDT9 knockdown cells and PC3-siRNA mock cells. However, the BAL1/ARDT9 mediated stimulation of STAT1-phosphorylation on Y701 observed in PC3 cells is weak and not comparable with the HR-DLBCL-cell line SUDHL7. The observed differences between the HR-DLBCL-cell line SUDHL7 and the metastatic prostate carcinoma cell line PC3 could be explained by the remaining IFN sensitivity of PC3 and DU145 cell lines. Phosphorylation of STAT1 on both S727 and Y701 cannot be further stimulated with IFNγ or IFNα in HR-DLBCL-SUDHL7 cells while in the PC3 and DU145 prostate cancer cell lines phosphorylation of STAT1 on Y701 is still further stimulated by IFNγ or IFNα. In order to test whether BAL1/ARDT9 has also a direct effect on cell proliferation and cell survival under normal growth conditions, we analyzed the proliferation of PC3-siRNA-BAL1/ARDT9 knockdown cells and PC3-siRNA-mock cells. Remarkably, these experiments revealed that knockdown of endogenous BAL1/ARDT9 in the metastatic prostate carcinoma cell line PC3 strongly inhibits proliferation and cell survival when compared with control cells (**Fig. 14A and B**). These preliminary results already indicate that BAL1/ARDT9, most likely together with STAT1, could also mediate survival and proliferation in prostate cancer and thus, contributing to development of metastatic prostate cancer.

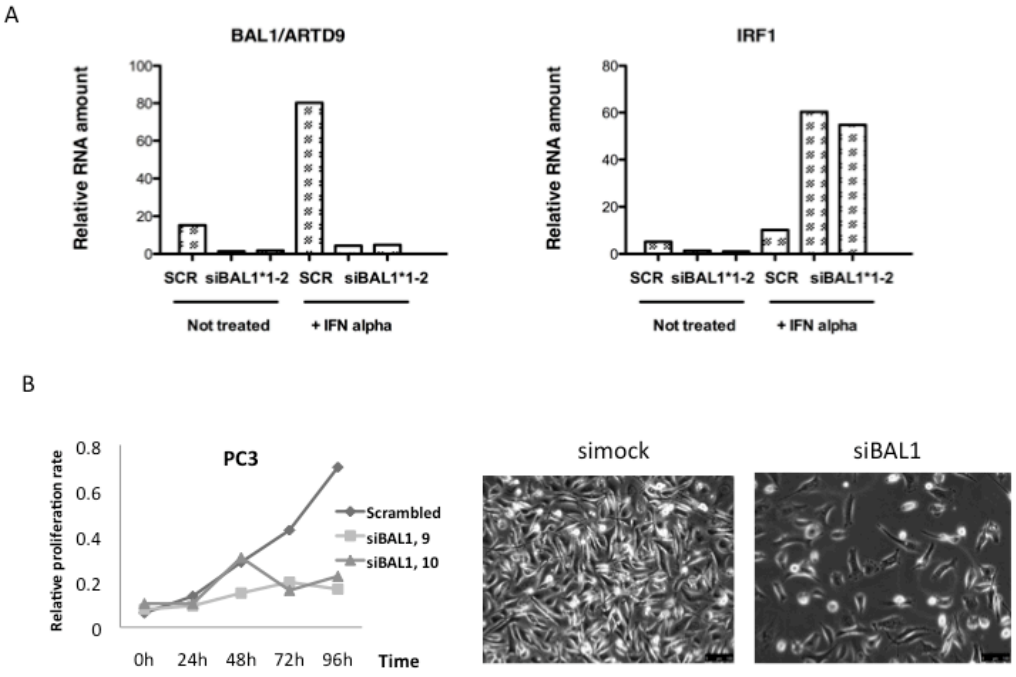


Figure 14. BAL1 inhibits IRF1 expression and mediate proliferation in metastatic prostate cancer cell line PC3.
A) qPCR analysis of IRF1 and BAL1 gene expression in PC3-parental, simock and siBAL1-knock-down cells treated without or with 1000 U/ml IFNγ for 8 h. **B)** Cell viability and proliferation analysis of PC3-parental, simock and siBAL1-knock-down cells in a time course of 96 h, was assessed by WST-1 Assay.

4.2.5 ARTD/PARP inhibitors negatively affect growth and survival of DLBCL

Many GCB and many NC DLBCL subtypes contain mutations in the *PTEN* gene. Recent studies showed that ARTD/PARP inhibitors negatively affect growth and survival of tumors harboring PTEN mutations (188). Moreover the observed interaction between BAL1/ARTD9 and STAT1 as well as between BAL1/ARTD9 and BAL2/ARTD8 and ARTD1, respectively, through their macro-domains seem to be dependent on mono-ADP-ribosylation (see result section, and personal communications H.C. Winkler and P.O. Hassa). These observations prompted us to speculate whether BAL2/ARTD8, ARTD1 or other enzymatically active ARTDs could be involved and also responsible for the observed effects, thus modulate

BAL1/ARTD9 mediated cell proliferation and survival in DLBCL. Besides inhibiting ARTD1 and ARTD2, DPQ, olaparib and ABT-888/veliparib can also inhibit the activity of BAL2/ARTD8, BAL3/ARTD7 or ARTD10, respectively (127, 172, 189). In order to test this hypothesis we treated different BAL1/ARTD9 non-expressing DLBCL cell lines and BAL1/ARTD9 expressing DLBCL cell lines with ARTD inhibitors. Indeed, our preliminary studies revealed that co-administration of the ARTD/PARP inhibitors olaparib (Astra Seneca), ABT-888 (veliparib, Abbott Laboratories) results in decrease of cell proliferation and increase in apoptosis in different DLBCL cell lines (an example is shown in Figure 15). However, no differences between BAL1/ARTD9 expressing and BAL1/ARTD9 non-expressing DLBCL cell lines could be observed under the tested conditions. Moreover, both *PTEN* wt (ABC-DLBCLs) cell lines and *PTEN* mutated (GCB-DLBCL) cell lines were negatively affected by ARTD/PARP inhibitors, indicating that neither BAL1/ARTD9 nor PTEN is required for the putative synthetic lethality induced by ARTD/PARP inhibitors. Interestingly, proliferation of Burkitt lymphoma cell lines (such as Ramos) seems not to be inhibited by ARTD/PARP inhibitors.

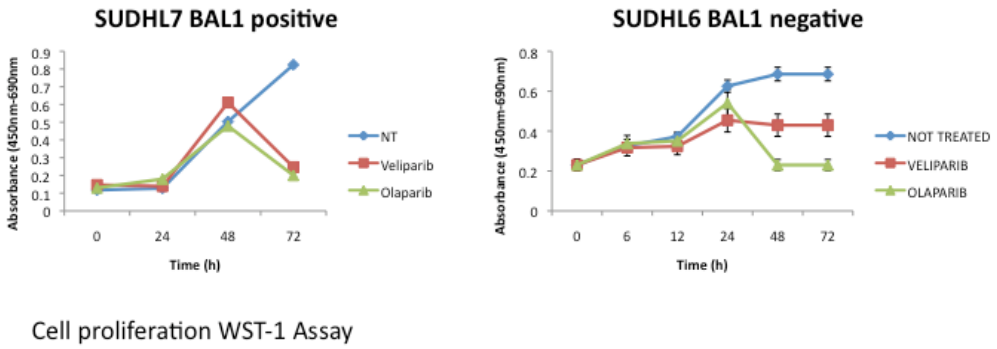


Figure 15 ARTDs inhibitors may impair the cell growth in non-Hodgkin's lymphoma cells.
Cell proliferation were analyzed using the WST-1 Assay.

5. DISCUSSION

5.1 Summary of the results in DLBCL

In this study, we identified BAL1/ARTD9 acting as a novel IFNγ-specific oncogenic survival factor, which inhibits the anti-proliferative and pro-apoptotic activities of tumor suppressor IRF1 while simultaneously activating the BCL6-mediated anti-apoptotic-pro-survival pathways. This study further shows that constitutive over-expression of BAL1/ARTD9 in DLBCL and androgen-refractory metastatic prostate cancer is tightly associated with constitutively active STAT1 signaling. BAL1/ARTD9 interacts with both STAT1 isoforms, STAT1α and STAT1β through its macro domains and together with STAT1β acts as a transcriptional repressor of IRF1 expression. Moreover this study showed first time that a risk related protein prevents the nuclear import or enhance the export of IRF1 in DLBCL. By counteracting the pro-apoptotic IFNγ-STAT1-IRF1 axes, BAL1/ARTD9 facilitates the oncogenic functions of STAT1 and explains why constitutive IFNγ-STAT1 signaling does not lead to apoptosis but rather to chemo-resistance in HR-DLBCL. These study also provides first evidence that BAL1/ARTD9 not only blocks IFNγ-STAT1-IRF1-mediated apoptosis and inhibition of growth, but could also enhance or lead to chemo-resistance in high-risk HR-DLBCL. Our observations could also provide a molecular mechanism for the risk-related activity of BAL1/ARTD9 in HR-DLBCL subsets without constitutive active STAT1 signaling. IFNγ induced expression of BAL1/ARTD9 could be directly involved in inhibiting the IFNγ-dependent host immune response against HR-DLBCL through negative feedback regulation of the extrinsic IFNγ-induced anti-proliferative and pro-apoptotic STAT1-IRF1 axes. Finally, the observed macro domain and mono-ADP-ribosylation-mediated interaction between BAL1/ARTD9 and STAT1 also indicates a regulatory

cross talk between BAL1/ARTD9 and other active members of the ARTD family in this process. Finally, our preliminary analysis of p53 negative and androgen-refractory metastatic prostate cancer cell lines already revealed that BAL1/ARTD9 could also play a similar survival related function in metastatic prostate cancer as observed in chemo-resistant HR-DLBCL. However many important questions remain:

5.2 Does BAL1/ARTD9 act both as a (co)activator and (co)repressor of IFNγ/STAT1 signaling in a context dependent manner in tumorigenesis ?

Previous studies showed that doxocyclin-induced over-expression of BAL1/ARTD9 in BAL1/ARTD9 non-expressing low risk GCB-DLBCL cells led to the induction of a very small subset of IFN-related genes, mainly type I (IFNα/β)-dependent genes (including IRF7 or OAS1-1) (25), thus providing preliminary evidence that BAL1/ARTD9 might act as an IFNα/β-dependent transcriptional coactivator (25). In sharp contrast, our studies showed that BAL1/ARTD9 does not transcriptionally activate these IFNα/β-related gene cluster in HR-DLBCL, but rather directly repress the pro-apoptotic IFNγ-STAT1-IRF-1 axes. These observed difference could be explained in two ways: Firstly, BAL1/ARTD9 could modulate gene expression in a cell type specific and context dependent manner; independent whether BAL1/ARTD9 is constitutively expressed or induced by IFNγ or IFNα/β in the corresponding cells. Secondly, the transcriptional activity of transiently over-expressed BAL1/ARTD9 in BAL1/ARTD9 non-expressing cells (i.e. induced by IFNγ or IFNα/β) is different from that of constitutively over-expressed BAL1/ARTD9 in HR-DLBCL or metastatic prostate cancer cell lines. Our results, together with the published data would even suggest a combination of both; transiently over-expressed endogenous BAL1/ARTD9 could activate IFNγ or IFNαβ-related gene expression in non-

expressing cells upon stimulation with IFNγ or IFNα/β, while subsequently terminating subsets of IFNγ or IFNαβ dependent gene expression programs and signaling pathways to prevent over-activation of IFNγ or IFNαβ signaling and eventually cell death. Thus constitutively expressed BAL1/ARTD9 in high risk HR-DLBCL and metastatic prostate cancer might just mimic in part the situation in primary cells under normal physiological condition and specifically terminates the subset of anti-proliferative and pro-apoptotic tumor suppressing IFNγ-dependent signaling pathways. The observed repression of IRF1 through BAL1/ARTD9 might also represent a negative feedback loop. A previous study provided evidence that transcriptional activation of BAL1/ARTD9 is mainly regulated by STAT1 and IRF1 (25).

5.3. How does BAL1/ARTD9 regulate the kinase activities in tumorigenesis?

Regulation of STAT1 phosphorylation is very complex. In the canonical IFN-gamma signaling pathway, activation of STAT1 is initiated through tyrosine phosphorylation on Y701, a process that involves JAK tyrosine kinases JAK1/2 and PYK2, which belongs to another PTK family (190, 191). JAK2 is thought to phosphorylate STAT1 on Y701 in the cytoplasm while JAK1 seems to be required for phosphorylation on Y701 in the nucleus, preventing nuclear export of STAT1 (190). STAT1, although phosphorylated on Y701, is unable to localize in the nucleus in the absence of JAK1 or JAK1 kinase activity (190). For full transcriptional activity and biological function, STAT1 must also be phosphorylated on S727 (192-194). STAT1 exists in two mayor isoforms, the full-length isoform STAT1α, which mainly acts as a sequence specific activator of gene expression and STAT1β, lacking the

transactivation domain and acting as a sequence specific transcriptional repressor (192, 195, 196). STAT1 phosphorylation on Y701 and nuclear translocation are required for IFN-induced S727 phosphorylation in canonical signaling pathways (193, 194). Phosphorylation on tyrosine 701 enhances the nuclear shuttling by triggering the nuclear retention of the shuttling of both isoforms STAT1α and β, which are kept in the nucleus until tyrosine dephosphorylation occurs (190, 193, 194, 197). However the molecular mechanisms underlying the IFN-induced S727 phosphorylation are not yet completely understood. Several kinases have been suggested to phosphorylate STAT1 on S727 in a cell type and stimuli dependent manner (192-194, 198-204). For instance, the multifunctional Ca(2+)/calmodulin-dependent kinase (CaMK) II can interact directly with STAT1 and phosphorylate STAT1 on S727 in response to IFN-gamma and Ca(2+) flux (199, 200). Other studies provided evidence that phosphorylation/activation of PKC-δ is required for phosphorylation of STAT1 on serine 727, as inhibition of PKC-δ activity blocks the IFNα/β-dependent serine phosphorylation of STAT1 and IFNα/β-inducible gene transcription (201, 202, 205). Furthermore, a recent study provided evidence that both the p38 mitogen-activated protein kinase (MAPK) and the CaMKII are activated in response to BCR signaling to converge on STAT1 phosphorylation on S727 for maximal gene expression (199). In contrast, UV irradiation-induced STAT1 phosphorylation on S727 seems to be exclusively p38 MAPK-dependent and independent of CaMKII or PKCs (198). More interestingly, recent studies found that STAT1 is constitutively phosphorylated on S727 in Wilms tumor by protein kinase Casein kinase (CK)-2 (203, 204). To make it even more complex recent studies provided evidence that nuclear translocation and serine phosphorylation of STAT1 on S727 can also occur independently of STAT1

tyrosine phosphorylation on Y701 (206). This situation is very likely the case in the metastatic prostate cancer cell lines PC3 and DU145.

Our studies provided first evidence that BAL1/ARTD9 directly or indirectly stimulates phosphorylation of STAT1 on Y701 but not S727 in HR-DLBCL. Since serine phosphorylation on S727 can only take place in STAT1α but not in STAT1β our results indicate that BAL1/ARTD9-mediated stimulation of tyrosine phosphorylation on Y701 could influence the shuttling kinetics of STAT1α and STAT1β, thus may tipping the balance between the STAT1α and STAT1β isoforms in HR-DLBCL and probably also in metastatic prostate carcinoma. BAL1/ARTD9 might therefore act as a cell and context specific regulatory factor in the fine-tuning of the IFNγ-dependent signaling cascades. Whether BAL1/ARTD9 directly or indirectly regulates the corresponding kinases involved in this process remains to be elucidated. The observed modulation of kinase activities may also be mediated by one of the interaction partners of BAL1/ARTD9, such as BBAP/DTX3L or BAL2/ARTD8. Indeed a recent study provided first evidence that ARTD8/BAL2 promotes the survival of myeloma cells by binding and inhibiting the kinase activity of c-Jun N-terminal kinase (JNK)-1 (160). Remarkably, JNK1 can also transcriptionally upregulate IRF1 and induce XIAP-associated factor 1 (XAF1) mediated apoptosis in gastrointestinal cancer (207).

Surprisingly, siRNA mediated knock-down of BBAP/DTX3L in the metastatic prostate cancer cell line PC3 led to an increase of phosphorylation of STAT1 on Y701 (personal communication SB. Bachmann and PO. Hassa), indicating that BBAP/DTX3L opposingly regulates the kinase activity of JAK1/2 in these cells. Thus, BAL1/ARTD9 together with BBAP/DTX3L and/or BAL2/ARTD8 may

synergistically or antagonistically regulate the phosphorylation pattern of STAT1 and IRF1 expression.

5.4. How does BAL1/ARTD9 regulate the subcellular localization of tumor suppressor IRF1?

Numerous tumor suppressors such as p53, shuttle between nucleus and cytoplasm in a tightly regulated fashion (208). Aberrant regulation of these processes in cancer cells can either inhibit export and leads to nuclear retention of the complexes or enhance the export of the corresponding tumor suppressor (208). Our study provides preliminary evidence that BAL1/ARTD9 is not only able to inhibit IRF1 on the level of transcriptional activation but also on the level of nuclear import or nuclear export. To the best of my knowledge this is the first observation that a risk related protein prevents the nuclear import or enhance the export of IRF1 in HR-DLBCL. However the molecular mechanism(s) underlying the observed IFNγ-dependent inhibition of nuclear import or enhancement of nuclear export of IRF1 remain(s) to be elucidated. One possibility might be that translocation of IRF1 is negatively regulated by the E3 ubiquitin ligase BBAP/DTX3L. BBAP/DTX3L has been previously suggested to regulate the sub-cellular localization of BAL1/ARTD9 (163). Complex formation between BBAP/DTX3L and BAL1/ARTD9 in the nucleus could facilitate the nuclear export of the BAL1/ARTD9 by BBAP/DTX3L (25) in HR-DLBCL.

5.5. Do BAL1/ARTD9 and BBAP/DTX3L together co-regulate the expression of IFNγ-dependent genes?

Since BAL1/ARTD and its binding partner the E3 ubiquitin ligase BBAP/DTX3L, are both constitutively over-expressed in chemo-resistant HR-DLBCL and metastatic

prostate cancer cell lines as well as their sub-cellular localization is tightly connected they might also function together in regulating IFNγ-dependent signaling and gene expression. BBAP/DTX3L and BAL1/ARTD9 have been suggested to form nuclear complexes (25). A recent study provided preliminary evidence that BBAP/DTX3L may shuttle to the nucleus in response to chemotherapy-induced DNA damage, selectively mono-ubiquitylate histone H4 lysine 91 and increase the protection of cells exposed to DNA-damaging agents (164). However the exact molecular mechanisms and potential connection to BAL1/ARTD remains to be elucidated. For instance, chemotherapy-induced DNA damage often also activates canonical and non-canonical IFNγ-dependent signaling pathways and induces an interferon-related gene signature for DNA damage resistance (120). Thus, it remains to be investigated whether BAL1/ARTD9 could recruit BBAP/DTX3L to (mono-ADP-ribosylated?) chromatin and whether (subsequent?) mono-ubiquitylation on histone H4 lysine 91 by BBAP/DTX3L could directly positively or negatively modulate the transcriptional activity of STAT1 and/or BAL1/ARTD9 on promoters of IFN-target genes co-regulated by STAT1 and BAL1/ARTD9.

5.6 Are STAT1 and STAT2 mono-ADP-ribosylated *in vivo*?

The observation that BAL1/ARTD9 interacts with STAT1 and STAT2 through their macro domains in an ADP-ribosylation dependent manner strongly indicates that the observed interaction depend on mono-ADP-ribosylation of STATs. Indeed, our initial in vitro screens showed that STAT1 could be mono-ADP-ribosylated in vitro by different ARTD family members, including ARTD10 (personal communication PO Hassa). Interestingly both BAL2/ARTD8 and ARTD10 are also constitutively expressed in HR-DLBCL and metastatic prostate cancer cell lines and also shuttling

to the nucleus in these cells (personal communications SB Bachmann & PO. Hassa). However we have no evidence so far found that BAL2/ARTD8, BAL3/ARTD3 or ARTD10 could also mono-ADP-ribosylate STAT1 or STAT2 in vivo.

5.7. Crosstalk or conditional/synthetic lethality between BAL1/ARTD9 and other ARTDs in tumorigenesis?

Both BAL1/ARTD9 and BAL2/ARTD8 not only interact through their macro-domains with STAT1, STAT2 (see result section) or STAT6 (132, 152) respectively, but also interact with other ARTD members such as ARTD1 and ARDT10 (personal communications H.C. Winkler and P.O. Hassa). Moreover, all three B-agressive lymphoma protein and macro-domain containing ARTDs BAL1/ARTD9, BAL2/ARTD8 and BAL3/ARTD7 can interact with each other through their macro-domains, (personal communications H.C. Winkler and P.O. Hassa). Interestingly, subsequent analysis revealed that the observed interactions are most likely dependent on (n)ADP-ribosylation (personal communications H.C. Winkler and P.O. Hassa). ARTD1 plays an important role as a tumor promoting factor in tumorigenesis (125). ARTD10 (former PARP10) has been initially identified as a novel c-Myc-interacting mono-ADP-ribosyltransferase that inhibits c-Myc- and E1A-mediated transformation (209). These preliminary results suggest a potential functional crosstalk among the B-aggressive lymphoma protein and macro domain containing ARTD family member BAL1/ARTD9, BAL2/ARTD8 and/or BAL3/ARTD7 and more interestingly between BAL1/ARTD9 and ARTD1. Indeed, our preliminary studies revealed that the ARTD/PARP inhibitors such as olaparib and ABT-888/veliparib, negatively affect cell proliferation and survival in different DLBCL cells. Remarkably the observed effects seems to be independent of any DNA repair deficiency in these cells since

both PTEN wt (ABC-DLBCLs) cell lines as well as PTEN mutated (GCB-DLBCL) cell lines were negatively affected by ARTD/PARP inhibitors.

On the other hand one has to be very cautious since no differences between BAL1/ARTD9 expressing and BAL1/ARTD9 non-expressing DLBCL cell lines could be observed under the tested conditions, indicating that neither BAL1/ARTD9 nor PTEN is required for the putative synthetic lethality induced by ARTD/PARP inhibitors in these cells. However these results suggest, at least in part, that a potential crosstalk among ARTDs could be the target of ARTD/PARP inhibitors in DLBCL cell lines. On the other hand since several recent studies clearly demonstrated that many ARTD/PARP inhibitors not only negatively affect the activities of most ARTD family members but also inhibit ARTD non-related enzymes such as the IFN/STAT1 dependent oncogenes and serine/threonine kinases PIM1 and PIM2 (210) it is difficult to speculate about the molecular mechanism underlying our preliminary observations. Remarkably, PIM kinase inhibition has been already proposed as a rational approach in lymphoma treatment (211). For instance, PIM1 is also over-expressed in GCB-DLBCL and cooperates with human BCL6 to promote the development of GCB-DLBCL (212). PIM2 over-expression has been also detected in subsets of mantle cell lymphoma, ABC-DLBCL, follicular lymphoma, marginal zone lymphoma-mucosa-associated lymphoid tissue type, chronic lymphocytic leukemia, and nodal marginal zone lymphoma cases (213, 214). PIM2 protein expression is also associated with an aggressive clinical course in ABC-DLBCL (213, 214). Thus, PIM1 and PIM2 kinase inhibition has been suggested to be a promising therapy for BCL6/PIM1-positive human GCB-DLBCL and PIM2-positive ABC-DLBCL, respectively (211-214). Future studies using a panel of single and double si/shRNA knockdown cells or novel highly-specific ARTD/PARP and JAK/STAT1 inhibitors with less or only few off

target effects will certainly give us more insight into the potential functional crosstalk between BAL1/ARTD9 and other ARTD family members in HR-DLBCL and other cancer types.

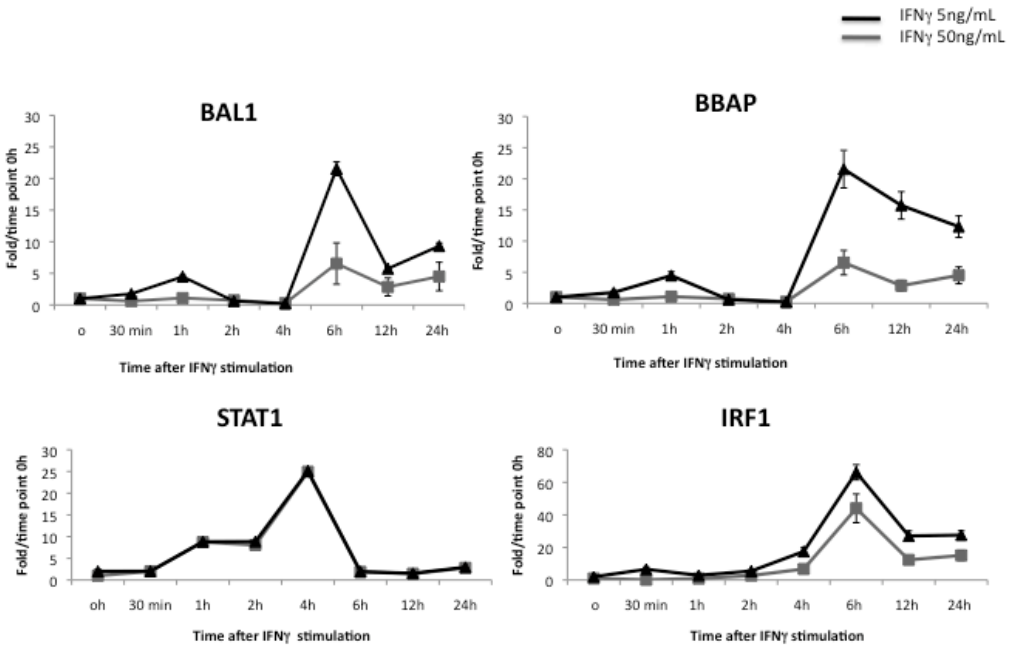
PERSPECTIVES

6.1. What are the functional roles of BAL1/ARTD9 under normal physiological conditions?

One of the most intriguing questions is how BAL1/ARTD9 could regulate IFNγ or IFNα/β dependent genes under normal physiological conditions. Mice models are one of the best approaches for analyzing the biological functions of a given gene product of interest. Several mice models for mono-ADP-ribosylating ARTD family members have been already established, including knockout mice of BAL2/ARTD8 and ARTD14/TiPARP (158, 215). However, no mice models for BAL1/ARTD9 are yet available, thus we can only speculate about the physiological roles of BAL1/ARTD9 in vivo. Interestingly, mice models for BAL2/ARTD8 already suggest that the macro domain containing ARTDs may play fundamental roles under normal physiological conditions. Several recent studies using BAL2/ARTD8 KO mice models provided first evidence that BAL2/ARTD8 regulates IL4 induced cell survival and proliferation of B cells, cytokine-regulated metabolic activity in B-cells as well as TH2 differentiation (152, 158, 216).

All three B-aggressive lymphoma protein and macro domain containing ARTDs BAL1/ARTD9, BAL2/ARTD8, BAL3/ARTD7 and their binding partner, BBAP/DTX3L are highly inducible proteins and only constitutively expressed in vivo at extremely low levels in lymphocyte-rich tissues (153). Expression of

BAL1/ARTD9, BAL2/ARTD8, BAL3/ARTD7 and BBAP/DTX3L is highly induced upon stimulation with IFNγ, IFNα/β, LPS (an example is shown in fig. 17) or upon infection with *H. pylori, S. typhimurium* and various RNA viruses (153-157). Thus BAL1/ARTD9 might also play important roles under normal physiological conditions (i.e. during cell differentiation) or in innate immunity. Moreover, since both BAL1/ARTD9 and BAL2/ARTD8 are acting as co-regulator of STAT1 (these study) and STAT6 (132, 152), respectively, IL-4, IFNγ and/or IFNα/β-dependent signaling pathways could be synergistically or antagonistically co-regulated by BAL1/ARTD9 and BAL2/ARTD8.



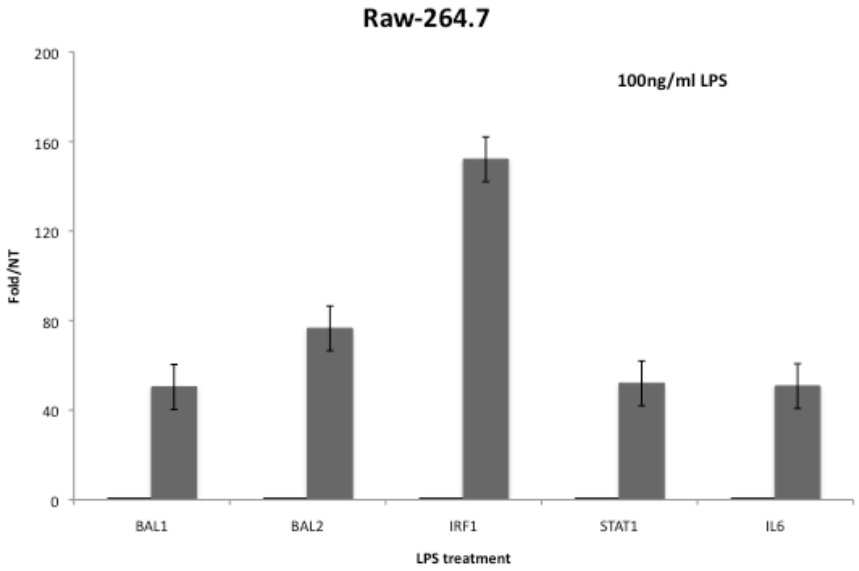


Figure 17. Relative mRNA expression levels of BAL1/ARTD9 in primary PBMCs and Raw 264.7 macrophages upon stimulation with IFNγ or LPS.
mRNAs were isolated from primary peripheral blood mononuclear cells (A) and RAW-264.7 cells (B) treated with or without IFNg (10ng/mL) in a time frame of 36 h (A) and 1h LPS (100ng/ml) stimuli (B). mRNAs levels of IFNs responsive genes were analyzed by qPCR and normalized against GAPDH.

6.2 Potential functional roles of BAL1/ARTD9 in innate immunity

Several studies have been recently shown that several ARTD family members, including ARTD3, BAL1/ARTD9, BAL2/ARTD8, ARTD10, ARTD11, ARTD12 and ZAP/ARTD13 are upregulated along BBAP/DTX3L, STAT1, OAS1-1, IRF1, IRF2, IRF7 and MX1 upon infection with bacterial and viral pathogens (154-157, 217). Moreover, three members of the WWE domain containing ARTD subgroup, namely ARTD11 and the zinc finger containing ZC3HDC1/ARTD12 and ZC3HAV1/ARTD13 have been recently identified as host factors with broad antiviral activities (156, 157). The exact molecular mechanisms underlying the antiviral activity of ARTD11 and ARTD12 are not yet known while the antiviral activities of ARTD13 are well investigated (218-221). ARTD13 directly interferes at multiple

levels with the replication of several important human RNA viruses, including SARS, influenza virus and human HIV-1 (218-221). For instance, ARTD13 can detect viral RNA and activates both the exosome-mediated RNA degradation pathway as well as RNA helicase RIG-I induced signaling cascades (218-223). Remarkably, most of the novel antiviral factors identified in the recent screening studies did only moderately inhibit the viruses when expressed individually (156, 157, 222). For instance ARTD11, ARTD12 and ZAP/ARTD13 showed only 20-30 % inhibition while the well-known antiviral genes, such as ISG20, MX2, IRF1, and OAS1-1 showed strong inhibition between 80-95% (157, 222). It has been already previously shown that expression of various combinations of antiviral factors have an additive inhibitory effect on viruses (156).

The observations that BAL1/ARTD9 interacts with ARTD11, ARTD12 and ZAP/ARTD13 (personal communications HC Winkler and PO Hassa) suggest that BAL1/ARTD9 might negatively or positively interfere with RNA virus replication or in general with the life cycle of RNA viruses. Moreover, BAL1/ARTD9 can also transcriptionally upregulate ARTD3, ARTD11, ARTD12 and ZAP/ARTD13 independent of IFNγ (an example is shown in Fig. 18). BAL1/ARTD9 and BBAP/DTX3L could act in combination with ZAP/ARTD13 as IFNγ and/or IFNα/β dependent antiviral factors. BAL1/ARTD9 could be required for terminating the IFNγ or IFN-α/β response upon infection with viral or bacterial pathogens. The regulation of IFNγ and IFN-α/β response must be tightly coordinated to maintain appropriate and timely immune reactions (224). Without mechanisms that shut down prolonged, inappropriate or excessive immune response and inflammation, the organism would die from damage caused by these viral or bacterial pathogens. Both pro- and anti-inflammatory signaling networks must be activated and balanced for an organism to

survive in the face of an infection with viral and/or bacterial pathogens that elicit an immune response (224). Therefore, BAL1/ARTD9 could not only co-activate the initial IFNγ and IFNα/β response as transcriptional co-activator but also subsequently or simultaneously inhibit the pathogen-induced IFNγ and/or IFNα/β dependent pro-apoptotic signaling pathways. Interestingly, most RNA virus induces cell death in order to further infect the surrounding tissues.

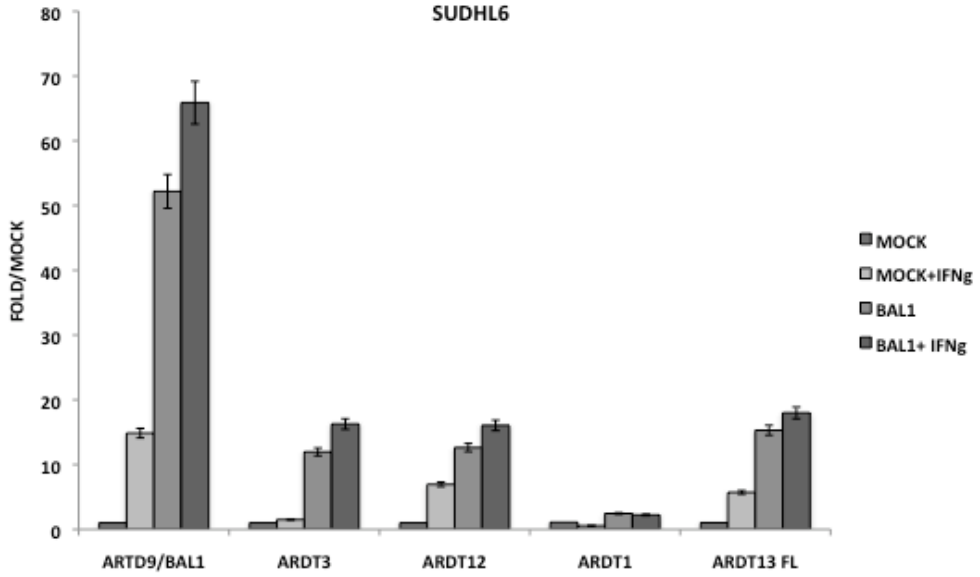


Figure 18. ARTD9/BAL1 enhances the mRNAs levels of others ARTDs with a known antiviral activity
mRNAs were isolated from BAL1/ARTD9 ectopically over-expressing and control (mock) cell lines treated with or without IFNγ (10ng/mL) ARTD genes were analyzed by qPCR and normalized against GAPDH.

6.3 Does BAL1/ARTD9 act as a regulator of STAT1-non-related signaling pathways?

The observation that expression of BAL1/ARTD9, BAL2/ARTD8, BAL3/ARTD7 and BBAP/DTX3L is highly induced upon stimulation with LPS or upon infection

with viral and bacterial pathogens suggests that BAL1/ARTD9 might also function as a regulator of STAT1 non-related signaling pathways such as NF-κB-dependent signaling. Moreover, interaction screen performed in the Hassa lab identified several NF-κB-dependent signaling related interaction partners of BAL1/ARTD9 involved in innate immunity, including p62/sequestosome. p62/sequestosome has been shown to play a central regulatory role in NF-κB related inflammation processes due to its modulator function in autophagy and as a crucial regulator and interaction partner of the atypical PKC kinases (225-228). Unfortunately initial gene expression screens did not reveal any NF-κB related co-activator or co-repressor function for BAL1/ARTD9 under the tested conditions (personal communication PO. Hassa), thus not supporting this hypothesis. On the other recent screens in the Hassa and Richards labs (CABMM UZH) using different in vitro models of STAT1/IFNγ and/or NF-κB-dependent mesenchymal stem cell (MSC) differentiation revealed a potential function of BAL1/ARTD9 in differentiation processes. STAT1 plays together with NF-κB, a critical regulatory role in both osteoclastogenesis and osteoblastogenesis (229-233). For instance, STAT1/IFNγ has been reported to act as a potent inhibitor of osteoclastogenesis while concomitantly induce osteoblastogenesis from MSCs both in vitro and in vivo, thereby modulating the balance between osteoclasts and osteoblasts in vivo (229-231, 234). No clear effect of STAT1 or IFNγ on adipogenesis has been so far reported. NF-κB-dependent signaling has STAT1/IFNγ-opposing effects in osteoclastogenesis and osteoblastogenesis (232, 233) while adipogenesis is inhibited by elevated NF-κB activity (235). Thus, osteoclastogenesis, osteoblastogenesis and adipogenesis from MSCs serves as a suitable in vitro model for investigating the potential functions of BAL1/ARTD9 in STAT1/IFNγ-dependent and independent cell differentiation processes.

Remarkably, these preliminary and ongoing studies already revealed that BAL1/ARTD9 might indeed act as a novel IFNγ and/or IFNα/β independent regulator of adipogenesis. Knockdown of BAL1/ARTD9 in human MSCs greatly enhanced adipogenesis (personal communications P.O. Hassa and A. Tiaden (CABMM, UZH)). Surprisingly, no effects were observed in osteoclastogenesis and osteoblastogenesis. Moreover, the observed effect in adipogenesis was not dependent on IFNγ and/or IFNα/β signaling (personal communications PO. Hassa and A. Tiaden (CABMM, UZH)). In contrary, the repressory activity of BAL1/ARTD9 strongly correlated with STAT5/Smad1/3 activities indicating that BAL1/ARTD9 might functionally interact or modulate the TGFβ-STAT5-SMAD1/3 signaling axes in adipogenesis (personal communications PO. Hassa and A. Tiaden (CABMM, UZH)).

6.4 Conclusions

This study identifies BAL1/ARTD9 as a novel IFNγ-specific oncogenic survival factor, which inhibits the anti-proliferative and pro-apoptotic activities of tumor suppressor IRF1 while simultaneously activating the BCL6-mediated anti-apoptotic-pro-survival pathways. Thus, by counteracting the pro-apoptotic IFNγ-STAT1-IRF1 axes, BAL1/ARTD9 facilitates the oncogenic functions of STAT1 and explains why constitutive IFNγ-STAT1 signaling does not lead to apoptosis but rather to chemo-resistance in HR-DLBCL and metastatic prostate cancer. In addition, our observations could also provide a molecular mechanism for the risk-related activity of BAL1/ARTD9 in HR-DLBCL subsets without constitutive active STAT1 signaling. BAL1/ARTD9 could be directly involved in inhibiting the IFNγ-dependent host immune response against HR-DLBCL through negative regulation of the extrinsic IFNγ-induced anti-proliferative and pro-apoptotic STAT1-IRF1 axes. Our

observations could also provide a molecular mechanism for the proposed activity of BAL1/ARTD9 in editing or inhibiting the IFNγ-dependent host immune response against HR-DLBCL (as previously suggested (25, 55, 89)), through negative regulation of the anti-proliferative and pro-apoptotic IFNγ-STAT1-IRF1 axes.

The observed macro domain and mono-ADP-ribosylation-mediated interaction between BAL1/ARTD9 and STAT1 also indicates a regulatory cross talk between BAL1/ARTD9 and other active members of the ARTD family in this process thus, could provide the rational base for the development of novel BAL1/ARTD9- and/or ARTD family member-specific inhibitors and activators, targeting either the catalytic domain or macro domains in HR-DLBCL. The combination of highly specific ARTD inhibitors with drugs targeting specifically STAT1 or the macro domains of BAL1/ARTD1 might be a strategy to increase the sensitivity of HR-DLBCL towards classical therapy, and thus pave the way to develop novel therapeutic strategies for the remainder of DLBCL patients suffering from aggressive chemo-resistant high-risk host response variants of DLBCL and chemo-resistant metastatic prostate cancers.

Finally, this study provides first insight how BAL1/ARTD9 may function under normal physiological condition in IFNγ-STAT1 dependent and independent processes, such as innate immunity against RNA viruses.

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July-Sept 2007	Exchange in USA • Harvard University (DEAS), Boston, MA
Sept 1999-June 2004	Secondary School • Liceo Scientifico E. Marini, Amalfi, Italy • Degree: Matura

Publication List

BAL1/ARTD9 represses the anti-proliferative and pro-apoptotic IFNγ-STAT1-IRF1-53 axes in diffuse large B-cell lymphoma

Rosalba Camicia,[1] Samia B. Bachmann,[1] Hans C. Winkler,[1] Marc Beer,[2] Marianne Tinguely[2] Eugenia Haralambieva [2] and Paul O. Hassa[1] (JCS, March 2013)

Running title: BAL1 inhibits IRF1

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